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Disarming and Sensing the Bacterial Type III Secretion System

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DISARMING AND SENSING THE
BACTERIAL TYPE III SECRETION SYSTEM

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

Miles C. Duncan

June 2015

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# TABLE OF CONTENTS

Abstract..................................................................................................................viii
Dedication and Acknowledgements.................................................................x
Chapter 1..................................................................................................................1

Chemical inhibitors of the type III secretion system: disarming bacterial pathogens

By Miles C. Duncan, Roger G. Linington, and Victoria Auerbuch

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Introduction: traditional antibiotics and bacterial resistance</td>
<td>3</td>
</tr>
<tr>
<td>Virulence Blockers</td>
<td>4</td>
</tr>
<tr>
<td>The Type Three Secretion System</td>
<td>6</td>
</tr>
<tr>
<td>T3SS Inhibitors: a recent history of discovery</td>
<td>11</td>
</tr>
<tr>
<td>Salicylidene Acylhydrazides</td>
<td>11</td>
</tr>
<tr>
<td>Other inhibitor classes</td>
<td>20</td>
</tr>
<tr>
<td>Targeted screens in <em>Yersinia</em></td>
<td>26</td>
</tr>
<tr>
<td>Structure-activity relationship studies</td>
<td>27</td>
</tr>
<tr>
<td>Conclusions and future directions</td>
<td>29</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>31</td>
</tr>
<tr>
<td>References</td>
<td>31</td>
</tr>
</tbody>
</table>

Chapter 2.........................................................................................................44

An NF-κB-based high throughput screen identifies piericidins as inhibitors of the

*Yersinia pseudotuberculosis* type III secretion system
By Miles C. Duncan, Weng R. Wong, Allison J. Dupzyk, Walter M. Bray, Roger G. Linington and Victoria Auerbuch

Abstract……………………………………………………………………………………………..45

Introduction………………………………………………………………………………………….47

Materials and Methods…………………………………………………………………………48

Results………………………………………………………………………………………………56

Screen to identify T3SS inhibitors………………………………………………………………57

Identification of piericidins with T3SS inhibitory activity………………………………….62

Mer-A 2026B and piericidin A1 do not inhibit Yersinia growth………………………………..65

Mer-A 2026B and piericidin A1 inhibit secretion of Yops in vitro………………………………67

Mer-A 2026B and piericidin A1 inhibit translocation of YopM………………………………70

Discussion…………………………………………………………………………………………..72

Acknowledgements………………………………………………………………………………77

References………………………………………………………………………………………….78

Chapter 3……………………………………………………………………………………………..84

Innate immune sensing of the type III secretion system

By Miles C. Duncan and Victoria Auerbuch

Introduction: Innate sensing by pattern recognition receptors……………………………..85

Sensing T3SS-mediated membrane perturbation……………………………………………87

Sensing T3SS structural components…………………………………………………………91

Sensing translocated T3SS cargo……………………………………………………………92

References…………………………………………………………………………………………94
Bacterial internalization is required to trigger non-canonical NF-κB activation in response to the bacterial type III secretion system

By Miles C. Duncan, Kevin S. Johnson, Joanne N. Engel, and Victoria Auerbuch

Summary........................................................................................................101
Introduction..................................................................................................102
Results...........................................................................................................106
Screen for inhibitors of T3SS-induced NF-κB activation...............................106
Identification of compounds that inhibit T3SS-mediated membrane disruption and Yop translocation.................................................................108
The *Y. pseudotuberculosis* T3SS triggers the non-canonical NF-κB pathway...112
Yop effectors that target the actin cytoskeleton shift the host cell NF-κB response .................................................................................................115
The *Pseudomonas aeruginosa* T3SS triggers non-canonical NF-κB..........117
Actin-targeting effectors prevent *Y. pseudotuberculosis* invasion of host cells..118
Extracellular bacteria do not activate non-canonical NF-κB.........................121
Intracellular and extracellular T3SS-positive *Yersinia* activate distinct host signaling pathways.................................................................121
Discussion....................................................................................................126
Experimental procedures...............................................................................136
Acknowledgements.....................................................................................141
References....................................................................................................143
LIST OF FIGURES AND TABLES

Chapter 1

Figure 1: The Yersinia type III secretion system………………………………………..7

Figure 2: Chemical structures of identified type III secretion system inhibitors……12

Table 1: Published type III secretion system inhibitors…………………………………22

Chapter 2

Figure 1: NF-κB-based HTS to identify small-molecule inhibitors of the Yersinia
T3SS…………………………………………………………………………………………..58

Figure 2: Identification of natural-product fractions that inhibit T3SS-driven NF-κB
activation but are not toxic to mammalian cells………………………………………..60

Figure 3: Piericidin A1 and the piericidin derivative Mer-A 2026B are the bioactive
constituents of prefraction 1772D……………………………………………………………..63

Figure 4: Piericidin A1 and Mer-A 2026B do not affect Y. pseudotuberculosis in vitro
growth…………………………………………………………………………………………66

Figure 5: Mer-A 2026B and piericidin A1 inhibit Yersinia type III secretion in vitro
more robustly than several previously identified T3SS inhibitors…………………..68

Figure 6: Piericidin A1 and Mer-A 2026B prevent translocation of YopM-Bla into
eukaryotic cells…………………………………………………………………………………71

Chapter 3

Figure 1. Current model T3SS sensing……………………………………………………88
Chapter 4

Table S1: Bacterial strains used in this study……………………………………….105

Figure 1: A Chemical Genetics Screen to Identify Chemical Inhibitors of the Yersinia
T3SS-mediated NF-κB response………………………………………………….107

Table 1: Compounds that inhibited Y. pseudotuberculosis Δyop6-triggered NF-κB
activation but not TNF-α induced NF-κB activation…………………………….…109

Figure 2: Effect of Inhibitors of T3SS-mediated NF-κB activation on Type III
Secretion……………………………………………………………………………110

Table S2: Compounds that inhibit either T3SS-mediated host membrane perturbation
or Yop translocation…………………………………………………………………111

Figure 3: Yersinia and Pseudomonas Lacking Actin-targeting Effectors Activate Non-
canonical NF-κB……………………………………………………………………..113

Figure 4: Yersinia Mutants that Cannot Target the Actin-Cytoskeleton are
Internalized…………………………………………………………………………119

Figure 5: Intracellular and Extracellular T3SS-positive Bacteria Trigger Distinct NF-
κB Pathways……………………………………………………………………………122

Table 3: Impact of select compounds on the Yersinia T3SS………………………..127

Figure S1: Gating strategy for flow cytometry-based translocation assay…………..134

Figure S2: LDH release assay………………………………………………………..135
ABSTRACT

Miles C. Duncan

Disarming and Sensing the Bacterial Type III Secretion System

The aims of my dissertation research were to determine how a bacterial virulence factor, the type III secretion system (T3SS), is sensed by the innate immune system and to identify small molecule T3SS inhibitors for use as biochemical tools and as potential therapeutics. The T3SS is a needle-like apparatus used by dozens of bacterial pathogens to inject effector proteins inside target host cells. This prevalent virulence factor allows bacterial survival and replication within the host, and T3SS-positive pathogens collectively cause more than 200 million illnesses each year. These T3SSs are highly conserved between bacterial genera, making them important for study and a potential drug target.

The first half of this work focuses on discovering T3SS inhibitors. In collaboration with the Linington lab and Chemical Screening Center, I developed a high-throughput screen (HTS) to identify small molecules that block T3SS formation or its proper function. This HTS was based on the host NF-κB response to the T3SS, and it uncovered piericidins as a novel family of T3SS inhibitors. These molecules are not toxic to bacteria or eukaryotic cells, and prevented type III secretion in vitro and T3SS-mediated effector translocation into host cells.

The second goal of my dissertation research was to determine how the T3SS triggers host immune responses, in particular the proinflammatory transcription factor NF-κB.
I carried out a chemical genetics screen to identify host-targeted small molecules that blocked the ability of eukaryotic cells to respond to the T3SS. I identified multiple pathways essential for this response, including five that prevented normal T3SS activity on host cells. As a result of this screen, I hypothesized and demonstrated that the T3SSs of intracellular and extracellular bacteria activate different immune responses. Specifically, intracellular bacteria trigger non-canonical NF-κB, an outcome not typically associated with innate immune sensing of pathogens. This finding could have broad implications for infections by intracellular pathogens that utilize a T3SS, as hyper-induction of non-canonical NF-κB is associated with rheumatoid arthritis and an increased cancer risk.
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Chapters 1, 2, and 4 of this dissertation are multi-author publications. I am responsible for all figures except: Figure 2 of Chapter 1, and Figures 3B, 3C, and 5B of Chapter 2.
CHAPTER 1

Chemical inhibitors of the type three secretion system: disarming bacterial pathogens

By Miles C. Duncan, Roger G. Linington and Victoria Auerbuch
The recent and dramatic rise of antibiotic resistance among bacterial pathogens underlies the fear that standard treatments for infectious disease will soon be largely ineffective. Resistance has evolved against nearly every clinically used antibiotic, and in the near future we may be hard-pressed to treat bacterial infections previously conquered by “magic bullet” drugs. While traditional antibiotics kill or slow bacterial growth, an important emerging strategy to combat pathogens seeks to block the ability of bacteria to harm the host by inhibiting bacterial virulence factors. One such virulence factor, the type three secretion system (T3SS), is found in over two-dozen Gram-negative pathogens and functions by injecting effector proteins directly into the cytosol of host cells. Without T3SSs, many pathogenic bacteria are unable to cause disease, making the T3SS an attractive target for novel antimicrobial drugs. Interdisciplinary efforts between chemists and microbiologists have yielded several T3SS inhibitors, including the relatively well-studied salicylidene acylhydrazides. This review highlights the discovery and characterization of T3SS inhibitors in the primary literature over the past 10 years and discusses the future of these drugs as both research tools and a new class of therapeutic agents.
INTRODUCTION: TRADITIONAL ANTIBIOTICS AND BACTERIAL RESISTANCE

One of the most pressing threats to the future of human health is the rapid and alarming evolution of antimicrobial resistance by pathogenic bacteria. Since the introduction of the first antibiotics, development of resistance has dependably followed clinical use, often in as little as three years (8). Currently 70% of hospital acquired infections are resistant to one or more antibiotics, with methicillin-resistant Staphylococcus aureus (MRSA) responsible for more U.S. deaths each year than HIV (8, 39). These substantial concerns are most pressing for Gram-negative bacteria for which only a single new agent has been approved in the last decade (57).

Despite a clear necessity for the development of new drugs, most large pharmaceutical companies have abandoned the field (10). The prevailing view among corporations like Glaxo SmithKline, Roche, and Eli Lilly is that research dollars are better invested in developing treatments that command high prices and require long courses of therapy (56). As expensive clinical trials and low success rates have made antibiotic research less profitable, Washington lawmakers are considering legislation like the GAIN Act for installing tax incentives, longer patents, and even federal funding to promote corporate innovation (55). Yet, it is unlikely any new classes of antibiotic drugs will reach the market within the next 10 years (9). Clearly a renaissance in antimicrobial research is needed to combat the emergence of multidrug-resistant and untreatable pan-resistant bacterial infections.
VIRULENCE BLOCKERS

In the past decade, a significant portion of academic antibiotic research has shifted from bacteriocidal or bacteriostatic drugs to virulence blockers (34). Unlike established antibiotics, virulence blockers inhibit pathogens by disarming the bacteria and preventing normal infection. These targeted virulence blockers inherently have clear benefits and disadvantages over conventional antibacterials. For example, traditional antibiotics are directed at widespread bacterial structures or processes required for growth. While this approach produces broadly effective drugs, these antibiotics indiscriminately kill both pathogens and members of the microbiota. Not surprisingly, disrupting the normal flora of the gut can have harmful side-effects, including increased risk of colitis caused by microbiota dysbiosis and Clostridium difficile over-colonization (7, 26). Additionally, recent research suggests that during antibiotic treatment, resistance arises in the abundant commensal flora, and this antibiotic resistance can then be passed on to more scarce pathogens in the gut through horizontal gene transfer (34, 39, 59, 60). Consequently, since the targets of virulence blockers are found only in a small subset of bacteria, they should apply selective pressure on fewer organisms than established antibiotics, and reduce the evolution and spread of antibiotic resistance genes.

Virulence blockers also should circumvent other common drug resistance pathways. For instance, some classes of virulence blockers target external processes, thus avoiding the common resistance avenues of drug efflux and diminished permeability (65). Additionally, these drugs may not promote a rapid rise of
resistance as they limit bacterial replication in the host but not in other environments, where antibiotic contamination from agriculture and animal farms can drive the evolution of resistance (34, 42). Though bacterial virulence mechanisms are diverse, anticipated progress in rapid infection diagnosis bolsters the potential for targeted therapeutic strategies. Already, several classes of inhibitors have been researched or even accepted into the clinic (8).

The most established virulence blockers are classified as antitoxins and are administered to counteract the secreted toxins of pathogens including Bacillus anthracis, Corynebacterium diphtheriae, and Clostridium tetani (8, 44, 61, 72). Often in the form of antibodies, these virulence blockers differ from most of the inhibitors currently being developed, but have been well-studied and used since the late 19th century (29, 58).

Recently, distinct molecules inhibiting Vibrio cholerae cholera toxin expression and biofilm formation have been explored (25, 53). Similarly, new work has examined the potential of inhibiting extracellular molecules and receptors involved in quorum sensing. Certain pathogens, including Pseudomonas aeruginosa, require sufficient bacterial numbers before forming biofilms or expressing virulence genes (22, 47, 64). Keeping these bacteria “blind” to their neighbors may be one strategy to allow easier immune clearance.

Targeting bacterial appendages is another potential method to reduce virulence, and specialized secretion systems are an obvious aim for researchers as they are required for growth in human hosts but not in other environments. While
much of this research has focused on type three secretion systems (T3SS), type two (T2SS) and type four secretion systems (T4SS) are also promising targets for study (4, 34). Both T2SSs and T4SSs translocate virulence factors across the bacterial membrane, but the T4SS mediates the translocation of genetic information as well (4). Thus, inhibiting T4S could play an important role in reducing the spread of antibiotic resistance. In addition to translocating virulence factors, T2SS are also responsible for assembling adhesive pili on the outer surface of bacteria (4). Many pathogens use their pili to attach to eukaryotic cells, and pilicides have been proposed as one method of reducing adhesion and thereby limiting infection (38, 54, 68).

THE TYPE THREE SECRETION SYSTEM

Type three secretion systems (T3SS) are required by dozens of animal and plant pathogens to cause disease, including the causative agents of plague, typhoid fever, and pneumonia (13). During mammalian infection, pathogens use the T3SS to inject effector proteins directly into target host cells, disrupting host defense mechanisms and allowing disease progression (5). In contrast, when the T3SS is nonfunctional, most T3SS-expressing bacteria are rendered benign and are easily cleared by the host immune system (5, 75). While not found in commensal bacteria (21), T3SSs of pathogens share many structural features, suggesting a single type of virulence blocker could inhibit T3S by multiple different bacterial species.

 Appropriately, a growing number of studies have explored T3SS inhibition as a therapeutic strategy for novel antibiotics. In a majority of these studies Yersinia spp.
Figure 1. The Yersinia type III secretion system. The T3SS is composed of a basal body, a needle structure, and a needle tip complex. The basal body spans the bacterial inner and outer membranes and is composed of proteins including the secretin protein YscC and the ATPase YscN (43). Once the basal body is assembled, it becomes a
functional secretion system. The YscF needle subunit is then targeted for secretion through the basal body, and secreted YscF subunits assemble to form a hollow extracellular appendage extending 60 nm from the bacterial outer membrane (12). The LcrV needle tip protein is secreted next and assembles into a pentamer at the apex of the needle. Upon host cell contact, the hydrophobic translocator proteins YopB and YopD are secreted through the T3SS. The LcrV tip complex is then thought to insert a YopB and YopD heterooligomer into the host membrane, forming a pore. Finally, partially unfolded effector proteins are translocated through the fully assembled T3SS into the host cell’s cytoplasm, where they interfere with host defense mechanisms.
have served as the model organism due to their well-characterized T3SS and readily available tools for research. *Yersinia* spp. have long been considered important pathogens for study; three species of the genus are pathogenic to humans, including *Yersinia pestis*, the causative agent of plague (5).

Found only in Gram-negative bacteria, T3SSs span the inner and outer bacterial membranes and share remarkable structural similarity to flagella (12, 13). The membrane-bound portion is known as the basal body and consists of a number of proteins (Fig. 1) (12, 13). Notable basal body proteins include secretin, which forms a ring found in the outer membrane, and the ATPase known as YscN in *Yersinia* spp. (45). YscN sits at the base of the T3SS and is thought to remove molecular chaperones from effector proteins, facilitating effector protein translocation into target cells (12, 13).

The extracellular needle of the *Yersinia* T3SS consists of a 60 nm long hollow tube made up of 100-150 polymerized YscF subunits (12, 13). The needle tip is formed by LcrV, a hydrophilic protein thought to provide a scaffold for pore formation in host cell membranes (12, 13). The host cell membrane pore is formed by the insertion of two hydrophobic proteins, YopB and YopD, which are known to interact with LcrV (5, 12). Upon host cell contact, various effectors are translocated through the T3SS into the host cell cytosol where they target host cell signaling pathways (5, 75).

As is the case for many T3SS-expressing pathogens (13), expression and activity of the *Yersinia* T3SS is tightly regulated in response to external factors (16).
The main transcriptional regulator of T3SS genes in *Yersinia* is LcrF, which is expressed only at low levels at 26°C (11, 28). However at 37°C, the temperature inside a mammalian host, translation of LcrF is thermo-induced leading to increased expression of virulence genes (5, 11, 28). Further regulation is mediated by LcrQ, in conjunction with YopD and the YopD chaperone LcrH; these cytoplasmic proteins inhibit translation of effector proteins until host cell contact has occurred (16, 28). LcrQ is then rapidly secreted, de-repressing expression of the T3SS effector proteins (17). T3S can be artificially induced in a lab setting by growing *Yersinia* at 37°C and in media containing a low level of calcium, which may mimic host cell contact.

While the T3S injectisome is highly conserved among pathogens, the function and number of effector proteins varies between species. In *Yersinia pseudotuberculosis* six proteins serve as effectors: Yops E, H, J, M, O, and T (5). YopE, YopH, YopO and YopT all target proteins involved in the host’s actin cytoskeleton, leading to the inhibition of both phagocytosis and the formation of reactive oxygen species (43, 66). As a result, *Yersinia* can avoid being taken up and killed by professional phagocytes. YopJ downregulates the inflammatory response by blocking MAPK, NFκB, and IRF3 signaling pathways (43, 69). YopM is critical for virulence and modulates immune cell function (1, 33, 67, 79). Collectively the Yops allow *Yersinia* to evade innate immune defenses.

T3SS-mediated disruptions of phagocytosis and host immune responses are essential for the progression of *Yersinia* infection. In fact, *Yersinia pestis* mutants missing the T3SS are avirulent even if injected into a host’s bloodstream (75). T3S is
also necessary for the virulence of many other human pathogens including
Pseudomonas aeruginosa, Shigella flexneri, enteropathogenic Escherichia coli, and
Salmonella typhimurium (13, 75). Accordingly, antimicrobial strategies that block
T3S have become attractive alternatives to traditional bacteriocidal drugs.

T3SS INHIBITORS: A RECENT HISTORY OF DISCOVERY

SALICYLIDENE ACYLHYDRAZIDES
One of the earliest accounts of a T3SS inhibitor came in 2003 when a group from
Umeå University in Sweden announced the promise of salicylidene acylhydrazides
(Fig. 2, 1) (Table 1) (30, 31). This initial breakthrough came from an assay designed
to measure T3S through a reporter-gene construct (31). In their experimental set-up,
Kauppi et al. took advantage of the tight coupling of T3S and translation of effector
proteins in Yersinia pseudotuberculosis by fusing a luciferase gene to the YopE
promoter (31). Compounds that inhibited T3S would result in lower YopE translation
and reduced luminescence. This high-throughput assay was used to successfully
screen a synthetic library of 9,400 small molecules purchased from ChemBridge,
obtaining three confirmed hits including compound 1 (Fig. 2), a
Figure 2. Chemical structures of identified type three secretion system (T3SS) inhibitors. Each inhibitor is numbered in order of appearance in the text and labeled by its discovering author. The apparent chemical diversity among T3SS inhibitors suggests there are multiple targets for inhibition.
salicylidene acylhydrazide (31). Furthermore, the authors demonstrated the discovered inhibitors did not affect bacterial growth in vitro and concluded they were acting on T3S in some capacity (31). As T3SSs and flagella are closely related, the authors sensibly investigated the effect of T3SS inhibitors on motility, finding only compound 1 reduced *Yersinia* motility (31). By screening in a whole-cell model, Kauppi et al. confronted obstacles of cell permeability and metabolism head-on, yet the exact targets and mechanisms of compounds 1-3 remained uncertain and required future study (31).

Two years later the group published a follow-up paper characterizing the salicylidene acylhydrazides (50). This research sought to elucidate the results presented in their previous study: it had remained unclear whether the inhibitors were acting specifically on the T3SS or reducing effector levels by broadly lowering overall transcription or translation. To make this important distinction, the authors tested whether compound 1 reduced transcription of the LcrQ promoter, which is upregulated at 37°C but not controlled by LcrF (50). The authors found compound 1 did not affect expression of an LcrQ-lux fusion reporter, suggesting this compound does not generally inhibit transcription of temperature-inducible promoters (50). However, compound 1 did block secretion of T3SS effector proteins in both wild-type and LcrQ mutant *Yersinia* strains (50). The authors concluded that compound 1 specifically targets T3S (50).

In 2005, Gauthier et al. independently identified salicylideneanilides, compounds closely related to salicylidene acylhydrazides, in a high throughput screen
designed to measure T3SS inhibition in enteropathogenic *E. coli* (EPEC) (21). The authors screened a Maybridge library of 20,000 molecules, monitoring secretion of the EPEC effector protein EspB by an enzyme linked immunosorbent assay (ELISA) (21). Their primary salicylideneanilide (Fig. 2, 4) reduced the expression of T3SS-associated virulence proteins (Tir, EspB, EscJ, EscC, and EspC), demonstrated by western blot (21, 76). The group also used transcriptional fusions to confirm that compound 4 inhibited the transcription of virulence associated-promoters, while non-virulence promoters were unaffected (21). In contrast to findings by Kauppi et al., the compound caused no change in motility or levels of flagellin in EPEC (21).

These breakthroughs were noted by several groups studying *Chlamydia trachomatis*, an obligate intracellular pathogen that encodes a T3SS, but for which there is no genetic system to aid its study. In 2006 Wolf et al. and Muschiol et al. showed the salicylidene acylhydrazides discovered previously for *Yersinia* spp. were able to disrupt normal progression of the *Chlamydia* infectious cycle (46, 78). Both groups demonstrated the compound was able to prevent chlamydial differentiation and multiplication within mammalian cells in a dose-dependent manner when given at various stages of infection (46, 78). The authors also saw downregulation of T3SS genes by reverse-transcriptase polymerase chain reaction (RT-PCR) (46, 78). Wolf et al. included *Coxiella burnetii* in their study, an intracellular pathogen possessing a T4SS rather than a T3SS, and found this pathogen’s infectious cycle unperturbed by the T3SS inhibitor, demonstrating its specificity (78). Additionally the group showed that the compound’s effects on *Chlamydia* were reversible, further highlighting the
utility of T3SS inhibitors as research tools for notoriously hard to study intracellular bacteria (78). Indeed, just one year later a report was published proving the same class of T3SS inhibitors were effective against the related pathogen *Chlamydia pneumoniae* (3).

Surprisingly it was through research on *Chlamydia* spp. that led to one of the more curious discoveries about the salicylidene acylhydrazides – their inhibitory activity’s dependence on iron concentration. In 2007 Slepenkin et al. found that by adding iron to HeLa cells infected with *C. trachomatis* they were able to reverse the effects of the inhibitors in a dose-dependent manner, while Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$ had no discernable effect (63). Previously hydrazones were shown to chelate iron, and the authors demonstrated the salicylidene acylhydrazide they studied, INP0341, and an analogue, INP0406, were able to bind iron to some extent (63). However, since INP0406 had little inhibitory affect on *Chlamydial* intracellular growth, iron depletion caused by INP0341 might not fully explain the compound’s mode of inhibition (63). The authors proposed that the salicylidene acylhydrazides at least partially work by altering the intracellular iron available to *Chlamydia* or the host cell, though it initially remained unclear how or why iron may be necessary for T3S (63).

Two years later Layton et al. proposed that salicylidene acylhydrazides rely on iron restriction to inhibit T3S in *Salmonella enterica* serovar Typhimurium (37). Through whole genome transcriptome analysis, the authors found that in the presence of INP0403, a quarter of all highly upregulated genes were involved in iron
acquisition or transport, suggesting that their compound may be an iron chelator (37). The authors confirmed previous work by showing INP0403 restricted iron availability, and its inhibitory activity could be reversed by the addition of exogenous free iron (37). Despite these findings it remains unclear what role iron plays in salicylidene acylhydrazide-mediated T3SS inhibition, and further work is necessary to fully appreciate iron’s affects on T3S and T3S inhibitors.

In 2007, Hudson et al. first demonstrated the in vitro and in vivo effectiveness of salicylidene acylhydrazides against SPI-1, one of the two T3SSs in Salmonella enterica serovar Typhimurium (24). The authors showed that their salicylidene acylhydrazides did not affect Salmonella growth, yet decreased SPI-1-induced red blood cell lysis by 30-60% and reduced SPI-1-mediated invasion of HeLa cells (24). The group was the first to demonstrate that salicylidene acylhydrazides block virulence in vivo, using a bovine intestinal ligated loop model (24). After injecting three Friesian bull calves with Salmonella, the authors found bacteria pre-incubated with the compounds induced less enteritis and recruited less neutrophils to the infected loops than Salmonella alone (24).

Negrea et al. further explored the salicylidene acylhydrazides’ effects on T3S in Salmonella enterica serovar Typhimurium (49). Analyzing nine related compounds, the authors found that most prevented Salmonella intracellular replication and invasion into MDCK cells, while only one was able to prevent effector translocation without also blocking effector protein expression (49). Next the authors created LacZ fusion constructs against several notable T3SS proteins:
transcriptional activator protein HilA, translocon protein PrgH, and effector protein SipC (49). All three reporters demonstrated the expected high levels of transcription under T3SS-inducing conditions, but this transcription was severely reduced in the presence of the salicylidene acylhydrazides (49). However, the salicylidene acylhydrazides did not repress transcription of an effector protein, SipB, suggesting the compounds do not broadly lower all gene expression (49).

The authors also explored the effects of salicylidene acylhydrazides on motility (49). Two of the nine studied inhibitors reduced bacterial motility, with this finding corroborated by decreased surface expression of flagellae observed by immunoblotting (49). Similarly, Layton et al. saw most *Salmonella* flagellar genes were repressed 1.5-2 fold in the presence of a salicylidene acylhydrazide inhibitor (37). These findings support observations by Kauppi et al., but are in contrast to Gauthier et al., who saw no change in the motility of EPEC treated with their closely-related salicylideneanilide (21, 31).

In 2008, Veenendaal et al. tested salicylidene acylhydrazides on *Shigella flexneri*, finding the compounds capable of preventing T3S, HeLa cell invasion, and macrophage killing (74). The authors also studied T3SS needle assembly, demonstrating by electron microscopy the number of T3SSs per bacterium were decreased by 30-40% in compound treated samples (74). In addition, the authors measured needle length by electron microscopy, observing much shorter needles in compound-treated samples (74). This was the first publication to suggest the salicylidene acylhydrazides functioned by altering needle assembly.
Slepenkin et al. published an account of a salicylidene acylhydrazide protecting against infection \textit{in vivo}, working with the sexually transmitted pathogen \textit{C. trachomatis} (62). During the trial, the authors gave 51 mice ten vaginal treatments with a salicylidene acylhydrazide, INP0341, or diluent alone (62). The mice were infected with \textit{C. trachomatis} two days after the first treatment, and INP0341 or sham control treatments continued for five days after infection (62). The mice treated with INP0341 were 60% less likely to have a positive vaginal \textit{C. trachomatis} culture than control mice during the four-week observation period (62). INP0341-treated mice also had lower antibody titers to \textit{C. trachomatis} after four weeks, indicating salicylidene acylhydrazides may have promise as prophylactics for \textit{Chlamydia} infection (62).

In an attempt to characterize the effects of salicylidene acylhydrazides on \textit{E. coli}, Tree et al. completed a whole genome transcriptome analysis of the bacterium in the presence of the inhibitory compounds (71). They found repression of many virulence genes including all genes within the locus of enterocyte effacement (LEE), a conserved pathogenicity island, as well as Ler and PchA, regulators known to be involved in coordinate expression of virulence genes (71). Through their findings, the authors propose this class of inhibitors functions through repression of virulence genes or possibly horizontally acquired genes (71). Interestingly, they also found upregulation of flagellum expression, unlike observations from previous studies in other organisms (71).
Wang et al. reported their findings from a study aimed at identifying the protein targets of a salicylidene acylhydrazide in *E. coli* (77). Using an affinity column containing the salicylidene acylhydrazide ME0055, the group identified proteins from an *E. coli* cell lysate that bound the inhibitor (77). Three enzymes were the most likely targets of the compound: WrbA and Tpx, proteins involved in defense against oxidative stress, and FolX, a poorly characterized epimerase (77).

To further study these enzyme targets, the researchers deleted each of the three genes in both *Yersinia* and *E. coli*, and then examined the T3SS and transcriptome of each mutant (77). They found 27 genes affected by the deletions, with most involved in locomotion, motility, and localization of the cell (77). Interestingly flagellar genes were downregulated while T3SS genes were upregulated (77). This suggests the inhibitors may actually increase the efficiency of the enzymes to which they bind, allowing them to better inhibit T3S.

The group recently published a follow-up study providing insight into the binding of salicylidene acylhydrazides to Tpx from *Yersinia pseudotuberculosis* (19). The authors identified the most likely binding conformations for their salicylidene acylhydrazides, and found the Tpx binding pocket to be mostly hydrophobic (19). The researchers also used far-western blotting to demonstrate the compounds bind to Tpx dimers with higher affinity than Tpx monomers (19).

**OTHER INHIBITOR CLASSES**
The first published account of a T3SS inhibitor came in 2002. Caminoside A (5), a glycolipid from a marine sponge, was found to inhibit secretion by EPEC (40). Though obstacles in compound supply prevented further characterization of the molecule, its discovery signaled the beginning of a promising area of research.

Following the discovery of the salicylidene acylhydrazides, other groups began screening commercial compound libraries for inhibitors of T3S. In 2007, Pan et al. developed a novel screen to identify T3SS inhibitors in Yersinia, using natural and synthetic libraries, and two years later reported new inhibitors discovered using this screen (51, 52). Normally when Yersinia is induced to express the T3SS by increasing the temperature and dropping extracellular calcium levels, the bacteria enter a virulent stage and cease replication (51). Assuming T3SS inhibitors would allow bacterial growth during T3SS inducing conditions, the authors used a GFP-strain of Yersinia to identify the few compounds that would promote increased fluorescence (growth) in this environment (52). Two of the three discovered compounds (Fig. 2, 6, 7) reduced effector secretion by Yersinia and EPEC, while all three (Fig. 2, 6-8) inhibited T3SS-mediated HeLa killing by Yersinia (52). These compounds were structurally distinct from previously reported T3SS inhibitors and spanned a range of structural classes including thioether bridged bicyclic and polyheteroaromatic systems (52). Interestingly, each compound inhibited secretion of Yops E, D, and M, but with varying efficiency (52). The targets of these compounds remain unknown.
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Table 1. Published Typing Selection System Inhibitors
In 2008, Iwatsuki et al. screened for compounds that inhibited red blood cell lysis through T3S by EPEC using a library of microbial extracts isolated from Japanese soil samples (27). The group discovered six unique inhibitors (Fig. 2, 9-14) produced by Actinobacteria including guadinomines A-D and guadinomic acid (27). These compounds were neither antibiotic nor cytotoxic and showed dose dependent T3SS inhibition, but were not further characterized and their mechanism of action remains unknown (27).

Later that year, Felise et al. reported a new class of inhibitors identified after screening 92,000 small molecules against the Salmonella T3SS (18). The library contained molecules of synthetic and natural origin purchased from Chem Div, Biomol, Maybridge, IF Labs and Bionet (18). To set up their screen, the authors fused a phospholipase to a Salmonella effector protein, classifying hits as molecules that reduced the enzyme’s extracellular activity, and presumably its secretion (18). While the study found seven hits, only one, a thiazolidinone (15), did not affect transcription of the hilA operon, which encodes a transcriptional regulator of T3S machinery (18). This molecule was next shown to inhibit formation of needle complexes and block T3S by Yersinia (18). The compound also prevented Francisella from secreting virulence factors through its unusual secretion system related to type four pilus secretion systems (18). Intriguingly, compound 15 (Fig. 2) was able to block T2S as well, but had no effect on flagella or motility (18). The authors propose that their inhibitor targets the only shared protein between the T2 and T3 secretion systems, the outer membrane protein secretin (18). Thiazolidinone was the first T3SS inhibitor
shown effective against a plant pathogen, *Pseudomonas syringae* (18), underscoring that T3SS inhibitors have the capacity to block virulence in a wide range of T3SS-expressing pathogens.

Harmon et al. published an extremely thorough study detailing both the discovery and characterization of several new inhibitors (23). The authors developed a novel screen by fusing a *Yersinia* effector to β-lactamase (23). First, target HEp-2 cells were loaded with a fluorescent dye, CCF2-AM, that emits a lower wavelength of light upon cleavage by β-lactamase (23). After infecting the HEp-2 cells with bacteria containing β-lactamase-T3SS effector fusions, the authors measured translocation efficiency by determining the ratio of blue/green fluorescence within the HEp-2 cells (23).

After screening 100,000 small molecules, the authors found eight hits (Fig. 2, 16-23) that were not cytotoxic to HEp-2 cells or generally antibiotic towards *Yersinia* (23). To determine whether the compounds affected T3SS needle formation, the group examined compound-treated bacteria by immunohistochemistry (23). All compound-treated samples showed some fluorescent staining of both YscF and LcrV, though compounds 20 and 23 showed slightly reduced staining of the T3SS needle (23). General chemical cross-linking analysis of YscF indicated only one of the compounds (Fig. 2, 17) caused minor changes in needle structure (23). Interestingly, under T3SS-inducing conditions none of the compounds were able to prevent Yop synthesis or secretion into culture supernatants (23). Six of the eight (Fig. 2, 18-23) were, however, able to prevent YopE translocation into HEp-2 cells, while all eight
increased Yop leakage into the surrounding supernatant during HEp-2 infection (23). One compound (Fig. 2, 22) greatly reduced *Yersinia* adherence to HEp-2 cells, and five (Fig. 2, 17, 18, 20, 22, 23) inhibited effector translocation by *Pseudomonas* (23). These findings suggest the compounds function by altering pore formation on target cells, or by disrupting hydrophobic interactions occurring between the membranes of *Yersinia* and the host cell (23).

The same year Aiello et al. screened 80,000 compounds against the *Pseudomonas* T3SS, finding several confirmed hits (2). As in *Yersinia*, reduced T3S capability in *Pseudomonas* causes decreased expression of all T3SS operons (2). Accordingly, the authors fused the *luxCDABE* operon to a *Pseudomonas* effector gene, classifying screened compounds as hits if they caused reduced bacterial bioluminescence (2). While five compounds (Fig. 2, 24-28) were confirmed as inhibitors using distinct effector secretion assays, the phenoxyacetamide (Fig. 2, 24) was the most promising as it was the only compound to rescue CHO cells from T3SS-mediated cell death by *Pseudomonas* (2). The compound was also effective against *Chlamydia* and *Yersinia*, but with reduced potency (2). While the molecular target of compound 24 is not known, the results suggest this phenoxyacetamide acts directly on the T3SS apparatus as T2S was unaffected, general protein expression was not blocked, and the authors observed inhibition of T3S even if the compounds were added after T3SS assembly (2).

In 2011 Kimura et al. reported their T3SS inhibitor protected against *in vivo* infection (36). The authors screened a diverse collection of natural products including
Actinobacterial, fungal, and plant extracts for inhibition of red blood cell lysis by EPEC (36). Among the 10 original hits, aurodox (Fig. 2, 29) was selected due to its potent inhibition and history of previous study (36). In an animal experiment, none of the control mice infected with Citrobacter rodentium survived past day 18, including those treated with tetracycline (36). Yet all mice treated with aurodox survived the infection, and demonstrated reduced incidence of colitis (36). Kimura et al. observed that aurodox did not affect expression of the housekeeping gene GroEL, however earlier work has suggested the molecule may affect bacterial translation by binding to ribosomal elongation factor Tu (36). The target of aurodox remains unclear, and further study is warranted to determine whether aurodox inhibits T3S via an indirect mechanism.

TARGETED SCREENS IN YERSINIA

Unlike early T3SS inhibitor screens, a few recent studies have targeted specific proteins necessary for regulation of needle formation or effector translocation. Yersinia virulence regulator LcrF is essential for T3SS formation, and LcrF mutants are severely attenuated in mouse infection models (35). In 2009 Kim et al. identified a N-hydroxybenzimidazole as an inhibitor of LcrF-DNA binding (35). The following year the same group published a more thorough exploration of their LcrF inhibitors (Fig. 2, 30-65) (20). In addition to preventing DNA binding, the authors found the compounds able to reduce Yersinia virulence against macrophages
and in vivo in mouse lungs, although their compound was administered to the mice one day before infection (20).

Another recent study identified inhibitors of YscN, a Yersinia ATPase responsible for removing chaperones from effector proteins and energizing the translocation process through the T3SS (70). The group confirmed YscN mutants were fully attenuated in a mouse infection model, and developed YscN inhibitors by using computational screening of a virtual 3D database of small molecules against a model of the active site of YscN (70). Thirty-seven promising compounds were used in biological assays and the researchers found three (Fig. 2, 66-68) able to inhibit both YscN ATPase activity and secretion of YopE into bacterial culture (70). However, in an infection assay the authors were unable to find significant inhibition of cytotoxicity as Yersinia still caused HeLa cell rounding in the presence of the compounds (70).

Although the authors were unable to discover an effective YscN inhibitor, their concept shows promise for future research. While bacterial ATPases have long been dismissed as potential antibiotic targets, the authors propose their seemingly low 25% homology to related human enzymes should alleviate concerns of drug cross-reactivity (70). Additionally, the group’s computer-aided approach to compound screening is an unexplored avenue that may bear fruit in future T3SS inhibitor studies.

**STRUCTURE-ACTIVITY RELATIONSHIP STUDIES**
High throughput screening using live bacteria has led to several promising classes of T3SS inhibitors. However the modes of action and biologically active functional groups must be explored afterwards. To systematically identify these biologically active sites, researchers often perform quantitative structure-activity relationship (QSAR) studies by synthesizing focused libraries of molecules that are structurally similar to one or more of the lead compounds. Selecting compounds for these focused libraries by statistical molecular design ensures chemical diversity and enhances the information yield from QSAR studies.

Kauppi et al. used statistical molecular design to create compounds based on a 2-arylsulfonylamino-benzanilide (Fig. 2, 3) identified in their original screen, and were able to improve inhibitory activity from 68% to 91% (32). Similar studies on salicylanilides and salicylidene acylhydrazides showed strong correlations between theoretical QSAR models and experimental T3SS inhibition (14, 15). This work demonstrated that statistical molecular design coupled with QSAR and validation can be a successful strategy for lead optimization of T3SS inhibitors (15).

Even as compounds are altered to aid in their inhibitory activity, oral bioavailability must be considered to improve their potential as therapeutic drugs. Defined as the fraction of an orally administered dose of unchanged drug that reaches systemic circulation, oral bioavailability is important both to allow reasonable dosage of new drugs and promote patient compliance (41). For many years chemists aiming to improve oral bioavailability have focused on Lipinski’s rule of 5 (41). This guideline states all drugs with high bioavailability must at most only violate one of
the following rules; no more than five H-bond donors, no more than 10 H-bond acceptors, a molecular mass less than 500 Daltons, and an octanol-water partition coefficient less than five (41).

Yet in 2002 Veber et al. published an often-overlooked improvement on this set of rules (73). The study examined properties of a large number of drug-like molecules to determine which characteristics led to better systemic circulation, offering more concrete explanations for the observations noted by Lipinski. The group found the most important molecular qualities for bioavailability were having less than seven rotatable bonds, increased hydrophobicity, and reduced polar surface area (73). Clearly, as T3SS inhibitors are further studied and prepared to enter clinical trials, quantitative structure-activity relationships and oral bioavailability improvements must be considered and balanced with \textit{in vivo} efficacy and toxicity.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The emerging threat of multi-drug resistant bacteria has led researchers to explore virulence blockers as new classes of antibiotics. Much research has focused on inhibitors of the T3SS, which is appropriate given the lack of new antibiotics in development to combat Gram-negative infection and the ubiquity of T3SSs in Gram-negative pathogens. Thus far, discovered T3SS inhibitors have been chemically diverse, suggesting there are many different potential targets for T3SS inhibition. Consequently, T3SS inhibitors also have enormous potential as research tools, capable of helping microbiologists explore host-pathogen interactions involving the
T3SS. Despite an increased interest in T3SS inhibitors, only the salicylidene acylhydrazides have truly begun to be biologically characterized for their mechanism of action. If any of this class of virulence blockers is to reach the clinic in the foreseeable future, more work is needed to identify and optimize the drugs’ functions.

While further high-throughput screening to find new inhibitors is certainly necessary, it must be undertaken with thought and reflection prioritized over brute force. David Payne of Glaxo SmithKline observed that anti-bacterial high-throughput screens are five times less likely to yield inhibitors than any other therapeutic targets, emphasizing a need for rethinking the process (73). Whole-cell screening has proven more successful than target-based in vitro approaches, as chemists have struggled to transform hits from target-based screening into active agents in whole-cell systems (57). Though the majority of current FDA-approved antibiotics are derived from natural products, few T3SS inhibitor screens have taken advantage of this resource, opting instead to examine extensively overlapping chemical libraries selected for their drug-like qualities (48). Additionally, previous studies have utilized less than 0.1% of the information provided by large screens; ideally “hit” compounds should be compared with structurally similar but inactive compounds from the original screen, in preliminary structure-activity relationship studies, to learn why certain compounds are not biologically active (42).

As the rise of antibiotic resistance coincides with a shortage of antibiotic research, unique drugs and methods of treating bacterial infections must be considered. Virulence blockers should not only function as research tools, but as
novel antibiotics that do not promote the rapid evolution of resistance. The development of virulence blockers is a complex goal that requires the resources of both academia and the pharmaceutical industry, but when achieved should bear invaluable rewards for worldwide human health.

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CHAPTER 2

An NF-κB-based high throughput screen identifies piericidins as inhibitors of the *Yersinia pseudotuberculosis* type III secretion system

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ABSTRACT

The type III secretion system (T3SS) is a bacterial appendage used by dozens of Gram-negative pathogens to subvert host defenses and cause disease, making it an ideal target for pathogen-specific antimicrobials. Here we report the discovery and initial characterization of two related natural products with T3SS inhibitory activity that were derived from a marine Actinobacterium. Bacterial extracts containing piericidin A1 and the piericidin derivative Mer-A 2026B inhibited *Yersinia pseudotuberculosis* from triggering T3SS-dependent activation of the host transcription factor NF-κB in HEK293T cells, but were not toxic to mammalian cells. As the *Yersinia* T3SS must be functional in order to trigger NF-κB activation, these data indicate that piericidin A1 and Mer-A 2026B block T3SS function. Consistent with this, purified piericidin A1 and Mer-A 2026B dose-dependently inhibited translocation of the *Y. pseudotuberculosis* T3SS effector protein YopM inside CHO cells. In contrast, neither compound perturbed bacterial growth *in vitro*, indicating that piericidin A1 and Mer-A 2026B do not function as general antibiotics in *Yersinia*. In addition, when *Yersinia* was incubated under T3SS-inducing culture conditions in the absence of host cells, Mer-A 2026B and piericidin A1 inhibited secretion of T3SS cargo as effectively or better than several previously-described T3SS inhibitors, such as MBX-1641 and aurodox. This suggests that Mer-A 2026B and piericidin A1 do not block type III secretion by blocking the bacterial-host cell interaction, but rather inhibit an earlier stage such as T3SS needle assembly. In
summary, the marine-derived natural products Mer-A 2026B and piericidin A1 possess previously-uncharacterized activity against the bacterial T3SS.
INTRODUCTION

Over two dozen Gram-negative pathogens use type three secretion systems (T3SS) to cause disease, including the causative agents of plague, pneumonia, and typhoid fever (1). These pathogens collectively cause over 200 million cases of human illness and greater than half a million deaths worldwide each year (www.who.int) (2). The issue of antibiotic resistance is most pressing for Gram-negative bacteria, for which only one new class of antibiotics has been approved in the last 15 years (3, 4). While T3SS-expressing bacteria have historically been susceptible to a number of antibiotics, many antibiotic-resistant strains have recently been isolated (www.CDC.gov). As T3SSs are typically required to cause disease (1), this virulence factor represents a promising target for new antimicrobial compounds.

The T3SS is composed of a basal structure spanning the inner and outer bacterial membranes and a needle that extends from the bacterial surface (5). This structure acts as a molecular syringe that injects bacterial effector proteins directly inside target host cells. While the structure of the T3SS is relatively conserved among T3SS-expressing bacteria, the suite of T3SS effector proteins expressed by each group of pathogens is completely distinct (1). The Yersinia pseudotuberculosis T3SS has been extensively studied and is often used as a model for T3SS-expressing pathogens (6). In Yersinia, the T3SS translocon proteins LcrV, YopB, and YopD form a pore in the mammalian plasma membrane upon host cell contact, enabling translocation of effector proteins inside the host cell cytosol (7). Y.
*pseudotuberculosis* effector proteins YopH, YopO, YopT, and YopE block phagocytosis and the formation of reactive oxygen species, while YopJ, YopM, and YopK dampen innate immune signaling (8, 9).

Over the past decade a number of research groups have discovered small molecule T3SS inhibitors by high throughput screening (HTS) (6). These inhibitors are diverse in chemical structure and their mechanisms of action are almost universally unknown. As virulence blockers are attractive alternatives to traditional antibiotics (10-12), discovering and better understanding new T3SS inhibitors is an important goal for anti-infectives research. In this study, we describe a unique HTS of marine-derived natural products for T3SS inhibitors that takes advantage of the ability of the *Y. pseudotuberculosis* T3SS to trigger NF-κB activation in HEK293T cells, an activity that is dependent on YopB (13). If the T3SS is rendered nonfunctional, either through genetic or chemical means, host cell NF-κB activity remains at a basal level during infection. The two related small molecules discovered through this novel HTS block translocation of *Y. pseudotuberculosis* T3SS effector proteins into eukaryotic cells, but do not act as general *Yersinia* antibiotics or mammalian cell cytotoxins.

**MATERIALS and METHODS**

**Bacterial Growth Conditions.** Bacterial strains used in this paper are listed in Table 1. *Y. pseudotuberculosis* was grown in 2xYT (yeast extract-tryptone) at 26°C with
shaking overnight. The cultures were back-diluted into low calcium media (2xYT plus 20 mM sodium oxalate and 20 mM MgCl₂) to an optical density (OD₆₀₀) of 0.2 and grown for 1.5 hours at 26°C shaking followed by 1.5 hours at 37°C to induce Yop synthesis as previously described (13).

**Cell Lines.** HEK293T cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO₂. CHO-K1 cells were maintained in Ham’s F-12K nutrient mixture with Kaighn’s modification (F-12K) with 10% Fetal Bovine Serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO₂.

**Natural Product Library and Bioassay-Guided Fractionation.** A screening campaign for T3SS inhibitors was carried out using a marine natural products library. This library was generated from environmental sediment-derived marine microorganisms specifically from the class Actinomycetales, known for their prolific production of pharmacologically interesting secondary metabolites. Sediment samples were collected into sterile 15 mL Falcon tubes by SCUBA mostly from the West Coast of the United States. The supernatant was removed and sediment samples were plated onto Actinobacterial-specific isolation media with added anti-fungal and Gram-negative antibacterial agents by radial stamping with sterile cotton swabs. Morphologically distinct colonies were picked and re-plated on Difco Marine broth agar plates repeatedly until pure isolates were obtained. Isolated Actinobacterial
colonies were subjected to liquid media culturing using our standard fermentation conditions (*vide infra*), and cryopreserved as glycerol stock solutions at -80°C.

Frozen stocks of environmental isolates were streaked onto fresh Marine Broth plates (Difco, USA) and incubated at 25°C until discrete colonies became visible. Selected colonies were inoculated into 7 ml of modified saline SYP (mSYP) media (10 g starch, 4 g peptone, 2 g yeast extract and 31.2 g instant ocean in 1 L of distilled water) and the cultures stepped up in stages by first inoculating 2.5 ml of 3-day-old 7 ml cell cultures into 60 ml of mSYP (medium-scale), followed by inoculation of 40 ml of these 2-day-old medium-scale cell cultures into 1 L of the same broth (large-scale). All cultures were incubated at 26°C and shaken at 200 rpm.

Large-scale cultures were fermented for 7 days prior to chemical extraction. Twenty grams of pre-washed Amberlite XAD-16 resin (\(\text{CH}_2\text{Cl}_2, \text{MeOH} \text{and water}\)) was added to each large-scale culture, shaken for 2 hrs (200 rpm), and the resulting slurry filtered under vacuum through a glass microfiber filter (Whatman). The cells, resins and filter paper were extracted with 1:1 \(\text{CH}_2\text{Cl}_2/\text{MeOH}\) (250 ml) and the suspension shaken at 200 rpm for 1 hr. Organic extracts were filtered and concentrated to dryness *in vacuo*. Dried crude extracts were pre-fractionated by solid phase extraction chromatography (5 g C\(_{18}\) cartridge, Supelco, USA) using a stepwise MeOH/ \(\text{H}_2\text{O}\) gradient : 40 ml of 10%, 20% (fraction A), 40% (fraction B), 60% (fraction C), 80% (fraction D), 100% MeOH (fraction E) then 100% EtOAc (fraction F). Fractions A – F were concentrated to dryness *in vacuo*, then resuspended in
DMSO (1 ml) and aliquots of these DMSO stock solutions reformatted to 384-well plates prior to screening.

From the primary screening of crude pre-fractions, active hits were selected for peak libraries screening to identify the active constituent(s) within a particular crude pre-fraction. A 45 µl aliquot of pre-fraction DMSO stock was lyophilized and fractionated by C\textsubscript{18} reversed-phase HPLC (Phenomenex Synergi Fusion-RP, 10 x 250 mm column, 2 ml min\textsuperscript{-1} flow rate) using a MeOH/H\textsubscript{2}O (0.02% formic acid) solvent system. Each pre-fraction was run on a gradient specifically tailored to produce the most highly resolved chromatography. Eluent was collected into deep well 96-well plates using an automated time-based fraction collection method consisting of 1 min time slices, and subsequently concentrated to dryness in vacuo. Dried plates were resolubilized (10 µl DMSO per well), sonicated to ensure homogeneity, reformatted to 384-well format and subjected to secondary screening.

**High-throughput screen.** On day one, 3.75x10\textsuperscript{6} HEK293T cells were plated onto three 100x20mm tissue culture dishes (BD Falcon) and incubated at 37°C/5% CO\textsubscript{2}. On day two, the HEK293T cells were transfected with an NF-κB luciferase reporter plasmid (Stratagene) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. This plasmid contains an NF-κB binding site (5x GGAAAGTCCCCAGC) upstream of the luciferase gene. On day three, the transfected cells were pooled and 5x10\textsuperscript{4} transferred to each well of a 384-well plate. Each plate was centrifuged for 5 minutes at 290 x g. *Yersinia pseudotuberculosis*
overnight cultures were back-diluted into 2xYT media to an OD$_{600}$ of 0.2, and grown in a shaking incubator at 26°C for 1.5 hours. The cultures were then pelleted by centrifugation and resuspended in half the original volume of low calcium 2xYT media. The bacteria were transferred to a 384-well plate containing low calcium media plus pre-fractions from the natural product library or plain DMSO and incubated for 1.5 hours at 37°C. Immediately prior to infection, pre-fractions or DMSO vehicle control were added to the 384-well plate containing HEK293T cells by a pinning robot. A Janus MDT pinning robot (Perkin Elmer) was next used to transfer *Y. pseudotuberculosis* from the bacterial 384-well plate to the HEK293T plate at a multiplicity of infection (MOI) of 7. After four hours at 37°C/5% CO$_2$, the media was aspirated before adding a 1:1 Neolite-PBS solution. Plates were covered in foil, incubated for 5 minutes, and bioluminescence measured using an EnVision plate reader (Perkin Elmer).

We used *Y. pseudotuberculosis* lacking the six known T3SS effector proteins YopHEMOJT (Δyop6) for this screen because YopHEMOJT are not required for T3SS-dependent NF-κB induction and, instead, several Yops modulate NF-κB activation (8). A *Y. pseudotuberculosis* mutant lacking the T3SS translocon component YopB (Δyop6/ΔyopB) was used as a T3SS-negative control. *Y. pseudotuberculosis* Δyop6 in the absence of natural products triggered, on average, 12-fold greater luminescence than the *Y. pseudotuberculosis* Δyop6/ΔyopB mutant (data not shown).
**Purified T3SS Inhibitors.** Aurodox was purchased from Enzo Life Sciences, MBX1641 from ChemBridge, C22 from TimTec, and C15, and C24 were purchased from Princeton Biomolecular Research.

Piericidin A1 and its analog Mer-A 2026B were isolated from an actinomycetes strain RL09-253-HVS-A, isolated from a marine sediment sample collected at Point Estero, California at 50 feet depth by SCUBA. This actinomycetes strain was identified as a *Streptomyces* sp. based on the typical morphology of Streptomycetes that form dry powdery spores on agar plates and by sequencing the 16s rRNA gene (data not shown). From a large-scale culture (4 L) of the strain producing extract 1772, 0.33 g of pre-fraction D (pre-fractionation method as described above) was obtained. The active constituents were purified using C18 RP-HPLC (gradient of 58% to 88% MeOH:0.02% formic acid/H2O, 2 ml/min, Synergi 10μ Fusion-RP column, Phenomenex, USA, tr = 12.5 min for Mer-A 2026B and 30.5 min for piericidin A1) to give 1.6 mg of Mer-A 2026B and 2.7 mg of piericidin A1. ESI-TOFHRMS analysis predicted the molecular formulae C24H35NO3 and C25H37NO4 for Mer-A 2026B and piericidin A1, respectively. One-dimensional 1H NMR spectra (Fig. S1) were obtained using a Varian Unity Inova spectrometer at 600MHz equipped with a 5 mm HCN triple resonance cryoprobe. Spectra were referenced to residual solvent signals (δH 7.24 for d-CDC13).

**Mammalian Cytotoxicity.** HeLa cell staining was performed as previously described (14). Briefly, HeLa cells were incubated with microbial extracts for 19 hours and
stained with Hoechst for visualizing individual nuclei. The 10% of natural product fractions that most reduced HeLa nuclear counts were classified as cytotoxic to mammalian cells and excluded from follow-up. This top 10% of nuclei reduction correlated strongly with the effects of previously characterized cytotoxic compounds within the training set used by Schulze et al. (14). For unpurified natural product fractions (including 1772D), the mammalian cytotoxicity data was generated by Schulze et al. (14). The cytotoxicity data for purified piericidin A1 and Mer-A 2026B at \( \leq 250 \mu M \) was performed specifically for this study.

**Growth Curves.** Overnight cultures of wildtype *Y. pseudotuberculosis* IP2666 were back-diluted to an optical density (OD\(_{600}\)) of 0.2 and 100 µl added to each well of a 96-well plate. A total of 0.3 µl of DMSO or purified natural products were added to each well. Bacteria were grown at 23°C in 2xYT media or at 37°C in low-calcium media (T3SS-inducing conditions) and OD\(_{600}\) of the culture measured every 15 minutes for five or six hours using a VersaMax Tunable Microplate Reader (Molecular Devices). The 96-well plates were continuously shaken throughout the experiment. Additional growth curves were carried out by taking samples of bacterial cultures at 0, 3, 6, and 24 hours of growth, serially diluting, and plating for colony forming units (CFUs) (Sup. Fig. 5). Starting inoculums of 4x10\(^3\) or 1x10\(^6\) CFU/ml were used. The 26°C growth curves were carried out in 500µl of 2xYT media with continuous shaking, while the 37°C growth curves were performed in high calcium media (2xYT plus 5mM CaCl\(_2\)) to prevent induction of the T3SS and associated
growth restriction. All growth curves used DMSO at 0.3%, pericidins at ≤143µM, or kanamycin at 50µg/ml.

**YopM Translocation Assay.** A total of 6x10³ CHO-K1 cells were plated in each well of a 384-well plate in 70 µL of F-12K medium plus 10% FBS and incubated overnight. The following day, *Y. pseudotuberculosis* YopM-b-lactamase (YopM-Bla) reporter strain overnight cultures were back-diluted into low calcium 2xYT media to an OD₆₀₀ of 0.2, and grown in a shaking incubator at 26°C for 1.5 hours. The cultures were then transferred to a 384-well plate containing low calcium media and purified compounds or DMSO, and incubated for 1.5 hours at 37°C. Immediately prior to infection, the purified compounds or DMSO were added to the 384-well plate containing CHO-K1 cells by a pinning robot (Janus MDT, Perkin Elmer). The pinning robot was next used to transfer *Y. pseudotuberculosis* from the bacterial 384-well plate to the CHO-K1 plate at an MOI of 6. Five minutes following this transfer, the plate was centrifuged at 290 x g for 5 minutes to initiate bacterial-host cell contact and incubated for 1 hour at 37°C/5% CO₂. Thirty minutes prior to the end of the infection, CCF2-AM (Invitrogen) was added to each well, and the plate was covered in foil and incubated at room temperature. At the end of the infection, the medium was aspirated and 4% paraformaldehyde was added to each well for 20 minutes to fix the cells. The paraformaldehyde was then aspirated and DRAQ5 in PBS was added to each well. The monolayers were incubated at room temperature for 10 minutes, washed once with PBS, and visualized using an ImageXpress MICRO automated
microscope and MetaXpress analysis software (Molecular Devices). The number of YopM-Bla-positive cells was calculated by dividing the number of blue (CCF2-cleaved) cells by the number of green (total CCF2) cells. Data from three separate wells were averaged for each experiment.

**Type III secretion assay.** Visualization of T3SS cargo secreted in broth culture was performed as previously described (13). *Y. pseudotuberculosis* low calcium media cultures were grown for 1.5 hrs at 26°C. Purified compounds or DMSO were added and the cultures were switched to 37°C for another 2 hrs. Cultures were spun down at 13,200 rpm for 10 min at room temperature. Supernatants were transferred to a new eppendorf tube. Ten percent final trichloroacetic acid was added and the mixture vortexed vigorously. Samples were incubated on ice for 20 min and then spun down at 13,200 rpm for 15 min at 4°C. The pellet was resuspended in final sample buffer (FSB) + 20% DTT. Samples were boiled for 5 min prior to running on a 12.5% SDS-PAGE gel. Sample loading was normalized for bacterial density (OD$_{600}$) of each sample. Densitometric quantification of the bands was done using Image Lab software (Bio-Rad), setting the first DMSO-treated WT *Y. pseudotuberculosis* YopE band to 1.00.

**RESULTS**
Screen to identify T3SS inhibitors. To carry out our HTS screen (Fig. 1), we transiently transfected HEK293T cells with a plasmid encoding an NF-κB-inducible luciferase reporter gene and infected these cells with *Y. pseudotuberculosis* carrying a functional T3SS in the presence of marine-derived natural products or DMSO vehicle control. Bioluminescence intensity produced by the cultures was used as a readout of T3SS activity. The in-house natural products library used consisted of over 5,000 partially purified “pre-fractions” containing 2-20 Actinobacterially-derived small molecules per pre-fraction (14, 15). This library has been shown to contain compounds with specific antimicrobial activities (15), but has not previously been screened for T3SS inhibitors.

We performed the screen described above on 2,560 pre-fractions in duplicate. We identified 355 pre-fractions that reduced NF-κB-driven luminescence by two standard deviations (stdev) below the mean of the DMSO-treated control (Sup. Fig. 1). This pool of pre-fractions may contain T3SS inhibitors, but may also contain compounds toxic to eukaryotic cells, killing the HEK293T cells before robust NF-κB activation can be induced. For instance, the known cytotoxins staurosporine and gliototoxin lowered NF-κB-driven luminescence to 7- and 11-fold less than that induced by the Δyop6/ΔyopB T3SS-deficient strain, respectively (Fig. 2A). To exclude pre-fractions containing potently cytotoxic compounds, we eliminated 217 pre-fractions that reduced T3SS-driven luminescence to at least one stdev below the average luminescence produced by the Δyop6/ΔyopB-infected cells (Sup. Fig. 1). A
FIG 1 NF-κB-based HTS to identify small-molecule inhibitors of the *Yersinia* T3SS. *Y. pseudotuberculosis* *yop6* was added to a 384-well plate containing natural-product fractions or DMSO and incubated for 1.5 h in low-calcium medium at 37°C to induce formation of the T3SS. The same natural products were robotically added to a 384-well plate containing HEK293T cells expressing an NF-κB-inducible luciferase reporter gene. The induced *Y. pseudotuberculosis* cultures were used to infect the HEK293T reporter cells at an MOI of 7. Four hours later, NF-κB-driven bioluminescence was measured and served as a readout of T3SS function in the presence of natural products.
total of 92% of these eliminated pre-fractions also showed HeLa cell cytotoxicity in work by Schulze et al. (14), validating this approach.

Of the remaining 138 pre-fractions, we hypothesized that some may contain compounds with general antibiotic activity, blocking *Y. pseudotuberculosis* replication. In a separate study, Wong *et al.* carried out antibiotic mode of action profiling on the same natural products library using a panel of bacteria. Based on their findings, we eliminated four additional pre-fractions that inhibited *Y. pseudotuberculosis* growth in broth culture (15)(Sup. Fig. 1).

Finally, we eliminated 48 additional pre-fractions that caused HeLa cell cytotoxicity in the study carried out by Schulze *et al.* (14), but did not reduce NF-κB activity in HEK293T cells to the very low levels seen with staurosporine or gliotixin (Sup. Fig. 1). This discrepancy in toxicity may be due to cell types used (HeLa vs. HEK293T) or the timeframe of the experiments (19 hours vs. 4 hours). However, T3SS inhibitors that are also cytotoxic toward mammalian cells would be significantly less useful as T3SS research probes and pre-therapeutics. Therefore, we eliminated pre-fractions displaying such dual activity, leaving 86 pre-fractions in our final pool.

Estimating an average of 11 compounds per pre-fraction and one compound per bioactive pre-fraction as being responsible for T3SS inhibitory activity, the hit rate per compound is ~0.3%. This hit rate is within the range of previously published T3SS-inhibitor screens (16-21).
FIG 2 Identification of natural-product fractions that inhibit T3SS-driven NF-κB activation but are not toxic to mammalian cells. (A and B) HEK293T cells expressing an NF-κB-inducible luciferase reporter gene were infected with *Y. pseudotuberculosis* Δyop6ΔyopB (nonfunctional T3SS) or Δyop6 (functional T3SS) in the presence or absence of the cytotoxins gliotoxin and staurosporine (A) or prefraction 1772D identified in our HTS (B). Bioluminescence was measured as a readout of T3SS function. The averages and standard errors of the mean (SEM) from two independent experiments are shown. *, $P < 0.05$, and **, $P < 0.005$ (Student t test) relative to HEK293T cells infected with the Δyop6 strain and DMSO treated. (C) HeLa cells were incubated with DMSO, staurosporine, or prefraction 1772D for 19 h. Fixed cells
were stained for tubulin (green), actin (red), DNA (blue), and phosphohistone H3 (to indicate mitosis) (cyan).
Identification of piericidins with T3SS inhibitory activity. We selected 21 pre-fractions for further investigation by separating the small molecules within each pre-fraction using liquid chromatography–mass spectrometry (LC-MS) to generate ‘one-compound-one-well’ peak libraries for secondary screening. This approach provides mass spectrometric, UV absorbance, and retention time data for all active constituents, and permits direct identification of bioactive compounds from active fractions. We then used these peak libraries to repeat the experiment described above (Fig. 1) and identified individual constituents able to inhibit T3SS-driven NF-κB activation.

We focused on pre-fraction 1772D, which caused a 3.5-fold decrease in T3SS-driven NF-κB activation (Fig. 2B). In comparison to staurosporine, pre-fraction 1772D did not cause gross changes in HeLa cell morphology in the absence of bacteria (Fig. 2C), indicating that the compounds in this pre-fraction are not grossly cytotoxic to mammalian cells. Upon further separation, pre-fraction 1772D yielded four fractions corresponding to 31, 32, 43, and 44 minutes of retention time on the HPLC that exhibited significant inhibition of T3SS-driven NF-κB activation (Fig. 3A) and displayed tractable chromatography for compound isolation (Fig. 3B). We re-grew the Streptomyces sp. strain RL09-253-HVS-A that produced pre-fraction 1772D and re-isolated and purified the bioactive compounds. The structures of two related compounds found in fractions 1772D 31, 32, 43 and 44 were determined using a combination of NMR and MS experiments (Fig. 3C; Sup. Fig. 2-3). We identified one compound as the piericidin derivative Mer-A 2026B and the other as piericidin
FIG 3 Piericidin A1 and the piericidin derivative Mer-A 2026B are the bioactive constituents of prefraction 1772D. (A) Prefraction 1772D was fractionated by HPLC-MS, and the eluent was rescreened to identify the active constituents. Fractions from minutes 31, 32, 43, and 44 contained compounds that inhibited T3SS-driven NF-κB activation in HEK293T cells. The averages and SEM are shown. *, $P < 0.05$, and **, $P < 0.005$ (Student $t$ test) relative to HEK293T cells infected with the Δyop6 strain and DMSO treated from two independent experiments. (B) Chromatogram (HPLC trace) of prefraction 1772D. Bioactive 1772D fractions 31, 32, 43, and 44 are boxed.
and contained compounds with related UV absorbance profiles. mAU, milliabsorbance units. (C) Structures of piericidin A1 and the piericidin derivative Mer-A 2026B identified through standard MS and NMR analyses.
A1 (22-24). Piericidins have previously characterized activity as insecticides, vasodilators, and inhibitors of the mitochondrial NADH dehydrogenase and as general antibiotics against certain bacteria (22, 23, 25-27).

**Mer-A 2026B and piericidin A1 do not inhibit *Yersinia* growth.** To confirm that the piericidins did not affect bacterial replication, we performed growth curves of *Y. pseudotuberculosis* in the presence of the purified compounds at 26°C and 37°C and monitored bacterial growth by optical density (Fig. 4). Piericidin-treated *Y. pseudotuberculosis* grew as well or better than DMSO-treated bacteria at all tested concentrations up to 143 µM, in contrast to the known antibiotic kanamycin. We also performed more sensitive 24 hour growth curves by serially diluting and plating cultures after 0, 3, 6, and 24 hours of growth (Sup. Fig 4). We observed no difference in bacterial replication between DMSO or 143µM piericidin-treated *Y. pseudotuberculosis* at all time points. As expected, we could not recover any colony forming units (CFUs) from kanamycin-treated cultures at 3, 6, or 24 hours of growth.
Fig 4. Piericidin A1 and Mer-A 2026B do not affect Y. pseudotuberculosis in vitro growth. Wild-type Y. pseudotuberculosis was grown at 23°C or 37°C with continuous shaking in the presence of DMSO, kanamycin, or piericidins. The averages ± SEM of values from three independent experiments [calculated as follows: (OD600 compound treated)/(OD600 DMSO treated)] are shown.
**Mer-A 2026B and piericidin A1 inhibit secretion of Yops in vitro.** We evaluated the ability of *Y. pseudotuberculosis* to secrete effector Yops into broth culture in the presence of the piericidins or five previously identified, commercially available T3SS inhibitors (Sup. Fig. 5). MBX-1641 and aurodox were shown to reduce *in vitro* type III secretion by *Yersinia pestis* and *Escherichia coli*, respectively, and were chosen as positive controls. In contrast, C15, C22, and C24 were shown to inhibit translocation of effector proteins inside host cells, but not Yop secretion *in vitro* and were chosen as negative controls (16). We grew *Y. pseudotuberculosis* in the presence of purified compounds or DMSO for 2 hours at 37°C in the absence of calcium (T3SS-inducing conditions). We then precipitated secreted proteins from the supernatant and analyzed relative protein abundance using SDS-PAGE analysis.

Mer-A 2026B at a concentration of 71 µM reduced secretion of the T3SS effector YopE by 45% (p<0.02), while lower concentrations of inhibitor demonstrated a dose-dependent decrease in inhibition (Fig. 5). Piericidin A1 blocked type III secretion by 65% (Fig. 5B). MBX 1641 (17) and C15 (16) at a concentration of 71 µM also significantly reduced YopE secretion, although this inhibition was only 22-33% (p<0.05 and p<0.04, respectively). In our hands, C22 and C24 did not significantly reduce T3S, which was consistent with the original report (16). However, C15 did block *in vitro* secretion. Aurodox (21) did not significantly inhibit YopE secretion at the highest concentration used, 12.5 µM (Fig. 5B). We chose not to test Aurodox at 71 µM, as Kimura *et al.* found that concentrations above 12.5 µM were cytotoxic to *E. coli.*
Mer-A 2026B and piericidin A1 inhibit Yersinia type III secretion in vitro more robustly than several previously identified T3SS inhibitors. (A) WT Y. pseudotuberculosis was incubated for 2 h under type III secretion-inducing conditions in the presence of various concentrations of the piericidin derivative Mer-A 2026B or DMSO. The secretome was precipitated with trichloroacetic acid and analyzed by SDS-PAGE analysis. The intensity of the Coomassie blue-stained band consistent with the size of YopE was quantified relative to DMSO-treated Y. pseudotuberculosis. The identity of the indicated YopE band was confirmed by Western blotting (data not shown). (B) The experiment in panel A was repeated using
piericidin A1 and the previously identified, commercially available T3SS inhibitors C15, C22, and C24 (16) and MBX1641 (17) at a final concentration of 71 µM (data not shown) (16, 17, 21). Aurodox was used at a final concentration of 12.5 µM (21). The average inhibition of YopE secretion by the T3SS inhibitors compared to DMSO [(inhibitor-treated YopE band intensity)/(DMSO-treated YopE band intensity)] and SEM from 3 or 4 independent experiments is shown. *, P < 0.05, and ** P < 0.02 (Student t test) relative to DMSO-treated WT Y. pseudotuberculosis.
**Mer-A 2026B and piericidin A1 inhibit translocation of YopM.** To analyze the ability of the piericidins to block translocation of *Y. pseudotuberculosis* T3SS effector proteins, we measured the translocation of a plasmid-encoded YopM–β-lactamase (YopM-Bla) reporter protein inside CHO cells using the fluorescent β-lactamase substrate CCF2-AM (28). In this assay, the CHO cells are loaded with the CCF2-AM dye that normally fluoresces green. If the YopM–β-lactamase chimeric fusion is translocated into these cells, the dye is cleaved and the cells fluoresce blue, providing a quantifiable readout of T3SS-mediated translocation.

The piericidin derivative Mer-A 2026B significantly reduced YopM translocation into CHO cells at all concentrations (Fig. 6), ranging from 9 µM to 143 µM. The 71 µM concentration displayed the most robust T3SS inhibition, 75% (p<0.05). Piericidin A1 also significantly diminished YopM–β-lactamase translocation at concentrations of 36 µM and greater (Fig. 6C). These results validate that the piericidins identified through our screen inhibit T3SS effector translocation into eukaryotic cells.
FIG 6 Piericidin A1 and Mer-A 2026B prevent translocation of YopM-Bla into eukaryotic cells. CCF2-loaded CHO cells were infected with Y. pseudotuberculosis expressing a YopM-Bla reporter. The relative efficacy of YopM translocation was measured by quantifying the intensities of uncleaved CCF2 (green) and cleaved CCF2 (blue). Shown are representative images (A) and the average percentage of blue cells (those that were injected with YopM-Bla) out of the total green cells (those that took up CCF2) and SEM from 3 or 4 independent experiments (B). *, $P < 0.05$, and **, $P < 0.005$ (Student $t$ test) relative to DMSO-treated Y. pseudotuberculosis.
DISCUSSION

In this study, we screened 2,560 marine-derived extracts from our in-house natural products library and identified two previously undiscovered T3SS inhibitors: piericidin A1 and the piericidin derivative Mer-A 2026B. These compounds blocked the *Y. pseudotuberculosis* T3SS in three distinct assays without cytotoxic effects to *Yersinia* or mammalian cells.

T3SS inhibitors belong to a novel antibiotic class called virulence blockers, which are designed to prevent normal infection by disarming pathogenic bacteria. This is in contrast to traditional antibiotics, which kill both pathogens and commensals alike by targeting essential pathways such as cell wall synthesis or translation (11, 12). Proposed bacterial targets for virulence blockers include quorum sensing mechanisms, toxin expression, pili, and secretion systems (10). Many of these virulence factors, including T3SSs (1), are rarely expressed in non-pathogenic bacteria, so the majority of the microbiota should be unaffected by virulence-targeted treatment. This more narrow selective pressure may slow evolution of resistance to T3SS inhibitors (29). In support of this, recent evidence suggests that resistance to traditional antibiotics often arises in the abundant commensal flora, and is then horizontally transferred to the more scarce pathogens (12, 30).

The HTS reported here is the first T3SS inhibitor screen that uses the host immune response to measure T3SS function (6). *Y. pseudotuberculosis* induces NF-κB activation in HEK293T cells dependent on expression of a functional T3SS (13). While the mechanism behind this NF-κB activation remains unclear, several bacterial
genetic requirements have been resolved. A *Y. pseudotuberculosis* Δ*yopB* mutant expresses T3SS injectisomes on its surface, but cannot make YopB-dependent pores on host cell membranes and, therefore, cannot facilitate translocation of T3SS cargo inside target host cells. Importantly, a Δ*yopB* mutant does not trigger NF-κB activation (13). Therefore, NF-κB activation in HEK293T cells can be used as an indicator of whether the *Y. pseudotuberculosis* T3SS is functional. We reasoned that small molecules that inhibit T3SS assembly or YopB secretion would block NF-κB activation during *Y. pseudotuberculosis* infection of HEK293T cells. Using NF-κB activation as a readout of T3SS function in a HTS, we found two related compounds that (i) inhibit secretion of Yops *in vitro*, which requires assembly of the T3SS apparatus (31), and (ii) block translocation of YopM inside mammalian cells, which requires YopB-dependent pore formation (32). Using these orthogonal assays, we validated that our HTS can identify genuine T3SS inhibitors.

A benefit of our HTS design is the recognition and exclusion of small molecules that are cytotoxic to mammalian cells. We observed that compounds toxic to mammalian cells, such as staurosporine and gliotoxin, inhibit NF-κB activation in our HTS to a level below that induced by a Δ*yopB* mutant in the absence of inhibitors (Fig. 2A). This enabled us to differentiate generally cytotoxic compounds from those with putative T3SS inhibitory activity. In fact, 92% of natural product-containing fractions that inhibited NF-κB activation to a level below that induced by Δ*yopB* also caused HeLa cytotoxicity as measured in a separate study using the same natural
products library (14). This aspect of our HTS may be particularly useful for screening potent compound libraries not yet tested against mammalian cells.

Only one other published HTS for T3SS inhibitors has made use of nucleated cells, where the authors used a Yop-b-lactamase translocation assay similar to the one used in Fig. 6 to validate our HTS results (16). Two other studies, Iwatsuki et al. and Kimura et al., published HTSs analyzing host-pathogen interactions with anucleated cells, measuring T3SS-mediated red blood cell lysis (21, 33). One major advantage of the HTS presented here is the use of a relatively low MOI of 7. We found that using a low MOI enabled greater sensitivity for identification of bioactive compounds (data not shown).

Piericidins were first reported in the 1960s as insecticides and inhibitors of mitochondrial electron transport (25). Later, the piericidin derivative Mer-A 2026B was discovered and proposed to have vasodilator activity (22, 23). However, we observed no cell death or morphological changes to HeLa cells incubated with ≤250 µM piericidins for 19 hours (Fig 2C, unpublished data) (14). It is possible that piericidin A1 and/or Mer-A 2026B only impact the physiology of specific eukaryotic cell types, or that the cytological profiling study performed on HeLa cells incubated with these compounds was not sensitive enough to detect changes to mitochondrial electron transport. In addition, despite reports in the literature identifying piericidins as antibiotics (26), we observed no effect of either piericidin A1 or Mer-A 2026B on Y. pseudotuberculosis growth in broth culture (Fig. 4, Sup. Fig. 5). A separate study tested the 1772D pre-fraction containing piericidin A1 and Mer-A 2026B against a
panel of 15 bacterial pathogens and only found inhibitory activity against two Gram-positives, *Bacillus subtilis* and *Listeria ivanovii* (Wong and Lintoning, unpublished data) (15). This indicates that piericidin A1 or Mer-A 2026B do not broadly affect bacterial growth and have a specific impact on the T3SS in *Yersinia*.

Interestingly, piericidins and the previously discovered T3SS inhibitor aurodox both have pyridine rings (Fig. 3C, Sup. Fig. 4) (21). This moiety is relatively rare in nature and it is tempting to speculate that these compounds may share a common molecular target. While aurodox was effective against the *E. coli* and *Citrobacter rodentium* T3SSs (21), in our study aurodox did not significantly inhibit Yop secretion by *Y. pseudotuberculosis* (Fig. 5B). It is possible that if aurodox and the piericidins target the same molecular structure, aurodox is more active against T3SSs belonging to the SPI-2 family, such as that carried by *E. coli* and *Citrobacter*, while the piericidins are more active against Ysc-family T3SSs, such as that utilized by all three pathogenic *Yersinia* (1). Like the piericidins, aurodox has antibiotic activity toward Gram-positive bacteria, but is thought to stall ribosomes by binding to the active domain of elongation factor Tu (34). More work is needed to identify the molecular targets of the piericidins and aurodox in Gram-positive and T3SS-expressing Gram negative bacteria.

In addition to aurodox, we compared the relative ability of the piericidins and several previously-identified T3SS inhibitors to block Yop secretion *in vitro*. Mer-A 2026B and piericidin A1 demonstrated the most robust T3SS inhibition, reducing YopE secretion into the culture medium by 45% and 65%, respectively. That we did
not observe a complete block of type III secretion may suggest an indirect mechanism of inhibition. MBX 1641, originally identified as an inhibitor of the *Pseudomonas aeruginosa* T3SS by Aiello *et al.* (17), showed a modest but significant reduction in effector secretion. In contrast, only one of three compounds discovered by Harmon *et al.* inhibited type III secretion in our *in vitro* assay. We were surprised to observe this inhibition by C15, as Harmon *et al.*’s exhaustive study proposed that their compounds interfered with pore formation or host cell contact (16). One possibility for the discrepancy is our higher concentration of compound used in the assay (71 µM vs. 60 µM).

An obvious challenge for the field of virulence blockers is a lack of standardized drug activity assays. Traditional antibiotics stall growth or kill bacteria, therefore one can compare drug efficacy by assaying for the minimum inhibitory concentration (MIC) of growth. *In vitro* measurements of secreted effectors may be one useful tool for comparing efficacy among secretion system inhibitors. Other classes of virulence blockers require their own comparative assays, and appropriate dosing will need to be established early in animal studies.

As T3SSs are highly conserved among Gram-negative pathogens, one inhibitor could be broadly effective against a number of infections. In fact, the best-characterized T3SS inhibitors, salicylidene acylhydrazides, have been successful against six genera of bacteria (6). Conversely, narrow spectrum drugs are likely to be more useful in the near future as point-of-care nucleic acid testing enters the clinic and allows rapid, accurate, identification of infectious agents (35, 36). Since T3SS
inhibitors are less likely to generate resistance, they also have potential as prophylactics for humans or in livestock. Follow-up studies on piericidins will determine if these compounds can block type III secretion by other pathogens, and focus on identifying the molecular target(s). As flagella are evolutionarily related to T3SSs, it will be important to determine whether piericidins affect flagellar motility. Lastly, structure-activity relationship studies could increase the potency of piericidins while abrogating potential off-target effects.

In summary, we have discovered two small molecules, piericidin A1 and Mer-A 2026B, with anti-T3SS activity. These compounds blocked \textit{in vitro} secretion and translocation of T3SS effectors inside host cells, but were not toxic to mammalian cells or \textit{Yersinia}. Furthermore, the piericidins were more robust in blocking Yop secretion than several recently discovered T3SS inhibitors, justifying further study of this new class of T3SS inhibitor.

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CHAPTER 3

Innate immune sensing of the type III secretion system

By Miles C. Duncan and Victoria Auerbuch
Introduction: Innate sensing by pattern recognition receptors

The innate immune system functions as an early and essential line of defense against microbial infection. Pathogenic bacteria, viruses, fungi, and parasites produce non-self and altered-self molecules that alert the host of their unwelcome presence (Janeway, 1989). Collectively, the shared microbial structures and molecules are referred to as pathogen-associated molecular patterns, or PAMPS, while pathogen-induced processes are termed “patterns of pathogenesis” (Vance et al., 2009). To detect these danger signals, host cells employ pattern recognition receptors (PRRs) to sense and respond to the microbial threat. Important PRRs for bacterial infection include Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Blander and Sander, 2012). Many immunostimulatory molecules, like lipopolysaccharide (LPS), sensed by TLR4, and peptidoglycan, sensed by TLR2 and NOD1/2, are present in both harmful pathogens and commensal bacteria alike. As such, some researchers have proposed “microbe-associated molecular patterns” (MAMPs) a more appropriate term than PAMPS, reflecting the idea that even commensals can trigger a robust immune response if they stray from their normal niche (Ausubel, 2005).

PRR engagement leads to activation of immune signaling cascades. Most TLRs reside on the plasma membrane and signal through the adaptor protein MyD88 to activate mitogen-activated protein kinases (MAPKs) and NF-κB (Blander and Sander, 2012). NF-κB is a critical family of proinflammatory transcription factors which, when active, lead to the production of cytokines and control cell survival and proliferation (Hayden and Ghosh, 2011). In contrast, NLRs are intracellular receptors
that alert the host when pathogens “violate the sanctity of the cytosol” (Lamkanfi and Dixit, 2009). The first NLRs discovered, NOD1/2, sense peptidoglycan and activate NF-κB through the adaptor RIP2. Several other NLRs, like NLRP, NLRC, and NAIP, form large complexes called inflammasomes in the cytoplasm. Inflammasome assembly leads to activation of caspase-1, which in turn cleaves immature IL-1β and IL-18 to allow their release (Lamkanfi and Dixit, 2009). These pathways have inherent crosstalk as well. For instance, Bergsbaken et al. demonstrated that Yersinia pseudotuberculosis activates NF-κB through TLRs, leading to pro-IL-1β and pro-IL-18 expression. These proteins are then cleaved to form mature cytokines and secreted upon T3SS-mediated inflammasome activation (Bergsbaken and Cookson, 2007). Inflammasome activation also leads to pyroptosis, an inflammatory form of programmed cell death dependent upon caspase-1 (Cookson and Brennan, 2001).

Unsurprisingly, pathogens devote significant resources to avoiding PRR recognition and dampening the subsequent immune responses. For instance, Yersinia pestis alters LPS on its surface to prevent TLR4 engagement, and employs T3SS effectors YopM, YopJ, and YopE, and the regulatory protein YopK to block NF-κB, MAPK, and inflammasome induction (Brodsky et al., 2010; Miller et al., 2005). However, the T3SS is sensed as an intrusive danger signal in and of itself, a further wrinkle in the host-pathogen arms race (Vance et al., 2009).

A growing body of research has aimed to determine how the innate immune system recognizes the T3SS, including Chapter 4 of this work. These studies have explored the PRRs involved, and attempted to ascertain which immune responses the
T3SS triggers through membrane perturbation, substrate translocation, and recognition of T3SS structural components.

**Sensing T3SS-mediated membrane perturbation**

Upon host cell contact, T3SS-positive bacteria insert translocon proteins (YopB and YopD for *Yersinia*) into the host cell membrane, forming a channel through which effector proteins can be translocated (Cornelis, 2006). This translocon insertion can lead to pore formation or host membrane perturbation, an attractive potential cause of immune responses. However, as Vance et al. wrote in 2009, “there is surprisingly little evidence that secretion systems themselves cause damage when expressed by wild-type bacteria at physiologically relevant multiplicities of infection (Vance et al., 2009). WT *Yersinia*, for instance, utilizes the Rho GTPase-targeting effector YopE and the regulatory protein YopK to minimize pore formation (Holmstrom et al., 1997; Mejia et al., 2008). Thus, reduced membrane perturbation by WT bacteria is likely harder to detect.

That same year, Auerbuch et al. published a study investigating how type III secretion triggers NF-κB in host cells lacking TLRs (as these receptors could activate immune pathways independently from the T3SS) (Auerbuch et al., 2009). The authors
Mammalian cells have evolved distinct but parallel mechanisms for innate immune recognition of the bacterial T3SS. Structural proteins (like the inner rod protein and needle subunit) are thought to be sensed by NAIPS leading to inflammasome activation. Hyperinjected translocon proteins are sensed by NLRP3, also activating the inflammasome. Actin cytoskeleton perturbation is sensed by NOD1, leading to induction of NF-κB and MAPK pathways. Less well understood is how membrane perturbation leads to NF-κB activation, which is the topic of Chapter 4.
found that an effectorless *Yersinia* strain, Δyop6, induced a robust NF-κB response, rivaling that of a strain containing four effectors, but lacking the NF-κB targeting effector YopJ. This NF-κB activation was independent of NOD1/2 and the caspase-1 inflammasome. These results suggested that *Yersinia* might trigger NF-κB through T3SS-mediated membrane perturbation or translocation of an unknown PAMP. To address this question further, the authors scrape-loaded type III secreted molecules into host cells by physically disrupting the host cells with a cell scraper, and did not observe an increase in NF-κB activity. However, they could not conclusively rule out NF-κB activation by T3SS-mediated translocation of an unknown PRR ligand. Several other pathogens activate NF-κB via the T3SS, indicating this response is not unique to *Yersinia* (Hii et al., 2008; LeBlanc et al., 2008).

Previous work by Gloria Viboud, Jim Bliska, *et al.* showed similar results (Viboud et al., 2006; Viboud et al., 2003). However, the authors primarily used a *Yersinia* strain containing effector Yops M and O, making it difficult to interpret whether their results represented immune recognition of membrane perturbation or a result of the two effectors. These studies did demonstrate that type III secretion could activate ERK, JNK, and p38 MAPK pathways (albeit in the YopM and O positive strain), and that these responses were reduced in the presence of YopE. YopE inactivates Rho GTPases, thereby reducing host membrane pore formation and cell death (LDH release) in response to the T3SS. Although somewhat unclear, these
results could point to T3SS-mediated membrane perturbation as a relevant trigger for NF-κB and MAPK induction.

A possible mechanism by which host cells might detect membrane perturbation is through ion flux. For instance, several studies have explored the role of potassium efflux in inflammasome activation. Arlehamn et al. found that *Pseudomonas aeruginosa* and *Salmonella typhimurium* T3SSs triggered the NLRC4 inflammasome, and this activation was dependent on extracellular potassium concentration (Arlehamn et al., 2010). Senerovic et al. demonstrated that for *Shigella*, host-membrane insertion of the translocon protein IpaB led to potassium flux, which activated caspase-1 (Senerovic et al., 2012). This activation was dependent upon the IPAF/ASC inflammasome, but not the NALP3 inflammasome. However, the authors used purified IpaB, which self-assembled and inserted into the membrane, making it unclear how relevant these results are to actual type III secretion. Munoz-Planillo et al. demonstrated that potassium efflux from the cytosol is sensed by NLRP3, leading to inflammasome activation (Munoz-Planillo et al., 2013). This method of inflammasome activation could be a pattern of pathogenesis, as several stimuli, such as nigericin and pore-forming toxins, can trigger inflammasome activation by allowing potassium efflux (Munoz-Planillo et al., 2013). However, Brodsky et al. found that adding extracellular potassium could not prevent *Yersinia* T3SS-mediated induction of caspase-1 (Brodsky et al., 2010), so this ion flux may only be important for recognizing certain T3SS-positive pathogens or certain types of membrane disruption.
Sensing T3SS structural components

As T3SSs are highly conserved between bacterial genera, it seems practical that the mammalian immune system would evolve methods of detecting related structural proteins shared by these pathogens. Accordingly, Miao et al. found that the NLRC4 inflammasome detects the basal body rod component of T3SSs from Salmonella typhimurium, Burkholderia pseudomallei, Escherichia coli, Shigella flexneri, and Pseudomonas aeruginosa (Miao et al., 2010). These rod proteins led to IL-1β release, and the authors proposed they share a sequence motif with flagellin, which is also detected by the NLRC4 inflammasome.

Interestingly, recent evidence shows the basal rod proteins are sensed by NAIPs, suggesting that NLRC4 may be an adaptor NAIPs interact with to activate the inflammasome. In 2011, Kofoed et al. found that NAIP2 was required for NLRC4 activation by PrgJ, the Salmonella inner rod protein. Suzuki et al. had similar results with NAIP2 and MxiI, with both groups confirming NAIP5 was required for flagellin-induced NLRC4 inflammasome activation (Suzuki et al., 2014). Several studies have also implicated the T3SS structural proteins in triggering the host immune system. Rayamajhi et al. and Yang et al. observed that NAIP1 could detect needle proteins from S. typhimurium, E. coli, S. flexneri, and Burkholderia spp. (Rayamajhi et al., 2013; Yang et al., 2013).

Surprisingly, Jessen et al. found that needle proteins from Yersinia, Salmonella, and Shigella can trigger TLR2 and TLR4, leading to MyD88 dependent
activation of NF-κB and/or activator protein 1 (AP-1), another proinflammatory transcription factor (Jessen et al., 2014). This somewhat contradicts the current paradigm that LPS activates TLR4 and peptidoglycan activates TLR2, and it remains to be seen if other groups can reproduce these results.

**Sensing translocated T3SS cargo**

The T3SS injects effectors and other cargo inside host cells, and these intrusive proteins are recognized as well. Perhaps because flagella and T3SSs are highly evolutionarily related, Sun *et al.* found that *Salmonella* injects flagellin into host cells (Sun *et al.*, 2007). Several groups have found this flagellin can trigger inflammasome activation (Miao *et al.*, 2006). For instance, Zhao *et al.* identified NAIP5 as the receptor that interacted with flagellin, leading to activation of the NLRC4 inflammasome (Zhao *et al.*, 2011).

Zwack *et al.* saw that hyperinjection of translocon proteins YopB and YopD is required for inflammasome activation by *Yersinia* (Zwack *et al.*, 2015). This work built upon the findings of Kwuan *et al.*, who used a *Yersinia* strain with a mutant YopD protein (lacking its transmembrane domain) to tease apart how the T3SS activated specific immune responses (Kwuan *et al.*, 2013). The authors found that while the mutant could form pores in macrophages, it could not translocate effectors or trigger IL-1β secretion, Egr1 expression, or TNFα expression. Their results suggested these specific immune responses required translocation of some unknown T3SS cargo, although the authors could not rule out the possibility that a WT
translocon was necessary for T3SS sensing. Given Zwack et al.’s recent findings, and work by other groups, this cargo may be YopB and YopD, and this may be the mechanism by which the YopK regulatory protein prevents inflammasome activation, as it minimizes YopB/YopD translocation into host cells (Brodsky et al., 2010; Dewoody et al., 2013; Zwack et al., 2015).

During infection, many T3SS-positive pathogens target the actin cytoskeleton as a means of cytosolic entry, or to avoid phagocytosis. Keestra et al. showed this manipulation, by Salmonella effector SopE, is sensed by the host cell (Keestra et al., 2013). Specifically, the authors found that SopE-mediated activation of Rho GTPases triggered the NOD1 signaling pathway, leading to RIP2-dependent NF-κB induction. This sensing of a common pattern of pathogenesis may be a generalized response to actin perturbation, as similar results were found for inhibition of actin polymerization (Magalhaes et al., 2005).

While we are yet to develop a clear picture of how the host recognizes this particular virulence factor, these studies have answered many outstanding questions in the field of innate T3SS sensing.
REFERENCES


Manipulation of small Rho GTPases is a pathogen-induced process detected by NOD1. Nature 496, 233-237.


apparatus through the NLRC4 inflammasome. Proc Natl Acad Sci U S A 107, 3076-3080.


CHAPTER 4

Bacterial internalization is required to trigger non-canonical NF-κB activation in response to the bacterial type three secretion system

By Miles C. Duncan, Kevin S. Johnson, Joanne N. Engel, and Victoria Auerbuch
SUMMARY

Infection of human cells with *Yersinia pseudotuberculosis* expressing a functional type III secretion system (T3SS) leads to activation of host NF-κB. Using chemical genetics, we show that the *Yersinia* T3SS activates distinct NF-κB pathways dependent upon bacterial subcellular localization. We found that wildtype *Yersinia*, able to remain extracellular, triggered NF-κB activation independently of the non-canonical NF-κB kinase NIK. In contrast, *Yersinia* lacking the actin-targeting effectors YopEHO, which becomes internalized into host cells, induces a NIK-dependent response and processing of the non-canonical NF-κB subunit p100/p52. Blocking actin polymerization and bacterial uptake using cytochalasin D shifted the host response from non-canonical to canonical NF-κB. We observed similar results using *Pseudomonas aeruginosa*, which expresses a related T3SS and the actin-targeting effector ExoT. Based on the identity of compounds that specifically inhibited T3SS-driven non-canonical NF-κB activation, we suggest that host cells detect T3SS-mediated vacuolar membrane perturbation, leading to a unique immune response.
INTRODUCTION

Bacterial pathogens and the mammalian innate immune system have co-evolved over millennia to recognize and combat one another. During infection, bacteria use virulence factors to survive and subvert host defenses, while the immune system employs pattern recognition receptors (PRRs) to identify and respond to these and other danger signals. PRRs, including Toll-like receptors (TLRs) and nod-like receptors (NLRs), sense pathogen-associated molecular patterns (PAMPs) like bacterial flagellin or lipopolysaccharide to activate appropriate inflammatory responses. These PRRs inhabit different cellular locations, allowing infected cells to further distinguish between cytosolic, endosomal, and extracellular insults (Blander and Sander, 2012).

One common virulence factor, which is also recognized as a PAMP, is the bacterial type III secretion system (T3SS) (Vance et al., 2009). The T3SS is a needle-like apparatus employed by dozens of bacterial pathogens to inject effector proteins into target host cells. Once inside, translocated effectors carry out species-specific functions, including dampening of immune signaling and prevention of phagocytosis by blocking actin polymerization. Many species of bacteria, including Yesinia and Pseudomonas aeruginosa, survive better extracellularly and actively target the actin cytoskeleton to prevent internalization. Effector translocation requires the translocon proteins YopB and YopD in pathogenic Yersinia (PopB and PopD in Pseudomonas aeruginosa), which are thought to insert in the host cell membrane and form a conduit
through which effector proteins are injected into the host cytosol (Cornelis, 2006). YopB and YopD were previously found to be important for the ability of *Y. pseudotuberculosis* to induce NF-κB activation in human cells (Auerbuch et al., 2009; Solomon et al., 2015; Viboud et al., 2003) suggesting that these translocon components stimulate host cell signaling in addition to enabling translocation of effector proteins (Zwack et al., 2015). This translocon-dependent NF-κB activation was independent of host TLRs, the NLRs Nod1 and Nod2, and the caspase-1 inflammasome (Auerbuch et al., 2009). Furthermore, T3SS effectors were dispensable for this response. In fact, the T3SS effector protein YopJ is known to dampen NF-κB signaling (Schesser et al., 1998).

NF-κB is a family of inducible mammalian transcription factors important for expression of inflammatory, developmental, and survival genes (Shih et al., 2011). The canonical NF-κB pathway controls gene expression through activity of the RelA/p50 heterodimer. This pathway is primarily involved in transient proinflammatory gene expression, while non-canonical NF-κB results in a slower, persistent response (Razani et al., 2011). Unlike canonical NF-κB, the non-canonical pathway is generally not associated with innate immune responses and results in nuclear translocation of heterodimerized RelB and p52 (Sun, 2011). This alternative pathway requires NF-κB inducing kinase (NIK), a central hub that integrates signals from various membrane receptors including B-cell activating factor receptor (BAFFR). Non-canonical NF-κB signaling is essential for regulating bone
metabolism, dendritic cell activation, B-cell survival, and lymphoid organogenesis (Sun, 2011).

To elucidate the host pathways essential for NF-κB activation following *Yersinia* infection, we used a chemical genetics approach, applying an annotated screening library of molecules with known bioactivity against eukaryotic cells to identify pathways required for type III secretion into host cells or the host cell response to the T3SS. Here we demonstrate that internalized T3SS-positive pathogens trigger a distinct NIK-dependent NF-κB pathway compared to T3SS-positive bacteria able to remain extracellular via actin-targeting effector proteins.
Table S1. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td><em>Yersinia pseudotuberculosis</em> IP2666 (no YopT expression)</td>
<td>Bliska et al. 1991</td>
</tr>
<tr>
<td>Δyop6</td>
<td>IP2666 ΔyopHEMOJ</td>
<td>Auerbuch et al. 2009</td>
</tr>
<tr>
<td>Δyop6/ΔyopN</td>
<td>IP2666 ΔyopHEMOJN</td>
<td>Auerbuch et al. 2009</td>
</tr>
<tr>
<td>WT+YopH-Bla</td>
<td>IP2666 pMM83::yopM-bla fusion</td>
<td>Adams et al. 2015</td>
</tr>
<tr>
<td>Δyop6+YopJ</td>
<td>IP2666 (MB202)</td>
<td>M. Bergman, unpublished</td>
</tr>
<tr>
<td>ΔyopM</td>
<td>IP2666 ΔyopM</td>
<td>Adams et al. 2015</td>
</tr>
<tr>
<td>ΔyopJ</td>
<td>IP2666 ΔyopJ</td>
<td>Adams et al. 2015</td>
</tr>
<tr>
<td>ΔyopE</td>
<td>IP2666 ΔyopE</td>
<td>Adams et al. 2015</td>
</tr>
<tr>
<td>ΔyopH</td>
<td>IP2666 ΔyopH</td>
<td>This work</td>
</tr>
<tr>
<td>ΔyopO</td>
<td>IP2666 ΔyopO</td>
<td>This work</td>
</tr>
<tr>
<td>ΔyopEHO</td>
<td>IP2666 ΔyopEHO (Contains YopJ and YopM)</td>
<td>This work</td>
</tr>
<tr>
<td>ΔexoUT</td>
<td><em>Pseudomonas aeruginosa</em> PA103 ΔexoUT</td>
<td>Garrity-Ryan et al. 2000</td>
</tr>
<tr>
<td>ΔexoU</td>
<td>PA103 ΔexoU</td>
<td>Garrity-Ryan et al. 2000</td>
</tr>
<tr>
<td>ΔpopBD</td>
<td>PA103 ΔpopBD</td>
<td>Kang et al. 1997</td>
</tr>
</tbody>
</table>
RESULTS

Screen For Inhibitors of T3SS-induced NF-κB activation

To identify host pathways required for *Yersinia* T3SS-induced NF-κB, we screened for compounds within the ICCB Known Bioactives Library that inhibit NF-κB activation in response to a *Y. pseudotuberculosis* mutant lacking the six known T3SS effectors YopHEMOJT (Δyop6), as these are dispensable for inducing NF-κB activation (Auerbuch et al., 2009) (Figure 1A). The ICCB library is a well-defined collection of 480 bioactive compounds with diverse eukaryotic targets. We used HEK293Ts expressing an NF-κB luciferase reporter, as these cells are readily transfected and lack TLR signaling pathways that could activate NF-κB independently of the T3SS (Auerbuch et al., 2009). We identified 126 compounds that were able to reduce T3SS-driven NF-κB activation by at least 2.5-fold (Supplementary Dataset 1). To eliminate compounds that broadly inhibit NF-κB signaling, we performed a secondary screen in which we examined inhibition of TNF-α-mediated NF-κB activation and excluded any compounds that reduced cytokine-driven NF-κB activity by more than 50% (Supplementary Dataset 1). Hits were ranked by degree of inhibition of T3SS-induced NF-κB activity compared to the degree of inhibition of TNFα-induced NF-κB (Table 1, Figure 1B) and the top 24 hits, 5% of the total library, were prioritized.

We reasoned that each of these 24 compounds could either block the activity of the T3SS on host cells, which is required for induction of NF-κB, or could block the ability of the host cell to respond to the *Yersinia* T3SS. Among the 24 hits were
Figure 1. A Chemical Genetics Screen to Identify Chemical Inhibitors of the *Yersinia* T3SS-mediated NF-κB response. (A) Flow chart of the screen to identify inhibitors of the T3SS-dependent NF-κB response. HEK293T cells expressing an NF-κB-luciferase reporter were infected with *Y. pseudotuberculosis* Δyop6 or treated with TNFα in the presence of compounds from the ICCB Known Bioactives Library and luminescence measured four hours post-inoculation. (B) The top 24 compounds that inhibited T3SS-mediated NF-κB activation more than TNFα-induced NF-κB activation were prioritized for further study. The average of two independent experiments ± standard error of the mean (SEM) is shown.
small molecules previously discovered to inhibit T3SS-mediated host membrane perturbation and T3SS effector protein translocation (cytochalasin D, PP1, PP2) (Mejia et al., 2008). In addition, several other compounds target host processes known to be important for the T3SS-host cell interaction such as latrunculin B, which, like cytochalasin D, targets the actin cytoskeleton, and cyclo [Arg-Gly-Asp-D-Phe-Val], a β1-integrin receptor antagonist that likely blocks the tight association between the bacterium and host cell important for efficient Yop translocation (Isberg and Leong, 1990; Yang and Isberg, 1993). Identification of compounds known to inhibit type III secretion into host cells serves as a proof of principle that our screening strategy is capable of identifying small molecules that disrupt the T3SS-host cell interaction.

**Identification of compounds that inhibit T3SS-mediated membrane disruption and Yop translocation**

It is possible that additional compounds among the 24 we identified interfere with T3SS activity (Yop translocation or membrane disruption) rather than the subsequent NF-κB response. Therefore, we directly tested 16 out of the 24 hit compounds for their ability to prevent membrane perturbation, or interfere with delivery of effectors into the host cell cytosol. Host membrane perturbation was monitored by visualizing ethidium bromide entry into host cells (Kwuan et al., 2013) and Yop translocation was assessed using *Y. pseudotuberculosis* expressing a YopH-β-(YopH- Bla) reporter fusion (Marketon et al., 2005) (Figure 2, Figure S1). Several compounds
Table 1. Compounds that inhibited *Y. pseudotuberculosis* Δopp6-triggered NF-κB activation but not TNF-α-induced NF-κB activation.

<table>
<thead>
<tr>
<th>Relative Inhibition of T3SS-induced NF-κB&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Compound Name</th>
<th>Molecular Target/ Description</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>116.45</td>
<td>TPEN&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Zinc and iron chelator</td>
<td>Ion/protonophores</td>
</tr>
<tr>
<td>41.17</td>
<td>Forskolin&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Blocks Protein Kinase A activation of Phospholipase C</td>
<td>Lipids, signal transduction</td>
</tr>
<tr>
<td>10.38</td>
<td>PP2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits Src-family tyrosine kinases, other kinases</td>
<td>Kinases</td>
</tr>
<tr>
<td>9.75</td>
<td>KN-62&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits CaM kinase II</td>
<td>Kinases</td>
</tr>
<tr>
<td>8.44</td>
<td>Cytochalasin D&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits Actin association and dissociation</td>
<td>Cytoskeleton dynamics</td>
</tr>
<tr>
<td>8.18</td>
<td>PP1</td>
<td>Inhibits Src-family tyrosine kinases, other kinases</td>
<td>Kinases</td>
</tr>
<tr>
<td>7.97</td>
<td>Wortmannin&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits Phosphatidylinositol 3-kinase (PI3-K)</td>
<td>Cytoskeleton dynamics</td>
</tr>
<tr>
<td>7.24</td>
<td>SB203580&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits p38 MAPK</td>
<td>Kinases</td>
</tr>
<tr>
<td>6.85</td>
<td>Genistein&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits tyrosine protein kinase, other kinases</td>
<td>Kinases</td>
</tr>
<tr>
<td>6.80</td>
<td>MY-5445</td>
<td>Inhibits Cyclic GMP phosphodiesterase, specifically PDE5</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>6.53</td>
<td>Cyclo [Arg...&lt;sup&gt;2&lt;/sup&gt;]</td>
<td>Antagonist of integrin avβ3</td>
<td>Cytoskeleton dynamics</td>
</tr>
<tr>
<td>5.79</td>
<td>Cloprostenol&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Agonist of prostaglandin receptor (PGF&lt;sub&gt;2α&lt;/sub&gt;)</td>
<td>Lipids, signal transduction</td>
</tr>
<tr>
<td>5.62</td>
<td>AG-1296&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits platelet-derived growth factor (PDGF) receptor</td>
<td>Kinase/phosphatase</td>
</tr>
<tr>
<td>5.46</td>
<td>SP600125&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits JNK</td>
<td>Kinase/phosphatase</td>
</tr>
<tr>
<td>5.13</td>
<td>Estradiol</td>
<td>Major estrogen, sex hormone and steroid</td>
<td>hormone</td>
</tr>
<tr>
<td>4.09</td>
<td>2-methoxyantimycin A3</td>
<td>Induces apoptosis</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>3.70</td>
<td>FPL-64176&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Activates Ca&lt;sup&gt;2+&lt;/sup&gt; channels (L-type)</td>
<td>Ion/protonophores</td>
</tr>
<tr>
<td>3.64</td>
<td>SDZ-201106</td>
<td>Opens sodium channels</td>
<td>Ion/protonophores</td>
</tr>
<tr>
<td>3.56</td>
<td>Swainsonine&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits α-mannosidases, glycoprotein processing</td>
<td>Glycosylation</td>
</tr>
<tr>
<td>3.29</td>
<td>Latrunculin B</td>
<td>Inhibits actin polymerization</td>
<td>Cytoskeleton dynamics</td>
</tr>
<tr>
<td>2.88</td>
<td>U-74389G&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits iron-dependent lipid peroxidation, antioxidant</td>
<td>Lipids</td>
</tr>
<tr>
<td>2.88</td>
<td>RHC-80267&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits Diaoyglycerol lipase, phospholipases C and A2</td>
<td>Lipids</td>
</tr>
<tr>
<td>2.58</td>
<td>Blebbistatin</td>
<td>Inhibits cell cycle progression, lowers affinity of myosin and actin</td>
<td>Cytoskeleton dynamics</td>
</tr>
<tr>
<td>2.56</td>
<td>Nocodazole&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Inhibits microtubule polymerization (β-tubulin)</td>
<td>Cytoskeleton dynamics</td>
</tr>
</tbody>
</table>

1. Fold Inhibition of T3SS-Induced NF-κB / Fold Inhibition of TNFα-induced NF-κB.
2. Purchased for further study.
Figure 2. Effect of Inhibitors of T3SS-mediated NF-κB activation on Type III Secretion.

16 of the 24 compounds selected in Figure 1 were assessed for their impact on (A) T3SS-mediated membrane disruption in 293T cells, measured by entry of ethidium bromide, and (B) T3SS-mediated effector translocation of YopH-Bla into 293T cells, measured by cleavage of the fluorescent dye CCF2. The average of three independent
experiments ± SEM is shown. *p < 0.05, **p < 0.01, ***p < 0.001, as determined by one-way ANOVA with Tukey’s HSD post-hoc test, where each indicated group was compared to the appropriate negative and positive controls: (A) ΔyopB + DMSO and Δyop6/ΔyopN + DMSO, or (B) ΔyopB+YopH-Bla + DMSO and WT+YopH-Bla + DMSO.

Table S2. Compounds that inhibit either T3SS-mediated host membrane perturbation or Yop translocation.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Inhibits Pore Formation</th>
<th>Inhibits Translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPEN</td>
<td>No Inhibition</td>
<td>25%</td>
</tr>
<tr>
<td>PP2</td>
<td>45%</td>
<td>40%</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>50%</td>
<td>40%</td>
</tr>
<tr>
<td>Cyclo[Arg…]</td>
<td>Not tested</td>
<td>30%</td>
</tr>
<tr>
<td>Forskolin</td>
<td>20%</td>
<td>No Inhibition</td>
</tr>
</tbody>
</table>
were incompatible with these assays due to competing autofluorescence or to interference with 293T cell adhesion (marked as N.D. in Figure 2). We identified 5/16 compounds that inhibited T3SS-mediated membrane perturbation and/or Yop translocation (Table S1). Three of these, PP2, cytochalasin D, and cyclo [Arg-Gly-Asp-D-Phe-Val], were expected, based on earlier studies (Mejia et al., 2008), but TPEN and forskolin have not been previously shown to inhibit T3SS activity on host cells. In contrast, 11/16 compounds inhibited T3SS-dependent NF-κB activation without blocking T3SS-mediated translocation or membrane disruption. These 11 small molecules are primarily annotated to interfere with host kinases, cytoskeleton dynamics, and lipid modification (Table 1).

The *Y. pseudotuberculosis* T3SS triggers the non-canonical NF-κB pathway.

The NF-κB luciferase reporter used in our studies is responsive to both canonical RelA/p50 and non-canonical RelB/p52 heterodimers (Wong et al., 2011). Because TNF-α triggers a largely canonical NF-κB response, our ability to identify compounds that block NF-κB activation in response to the *Yersinia* T3SS but not in response to TNF-α suggests that the T3SS triggers the non-canonical NF-κB pathway (Sun 2008). We confirmed non-canonical NF-κB activation by demonstrating enhanced processing of the precursor protein p100 to p52 upon infection of 293T cells with *Y. pseudotuberculosis* Δyop6 compared to WT and the ΔyopB mutant (Figure 3C). In addition, we used RNA interference to deplete cellular levels of NIK,
Figure 3. *Yersinia* and *Pseudomonas* Lacking Actin-targeting Effectors Activate Non-canonical NF-κB. (A) NF-κB luciferase reporter 293T cells were infected with WT and mutant *Yersinia* for four hours and luminescence measured. The *Y. pseudotuberculosis* Δyop6 strain triggered significantly more NF-κB than every strain except ΔyopJ. (B) The same experiment as in A was repeated, except expression of the non-canonical kinase NIK was knocked down using siRNA, or control siRNA was used. Data is expressed as NF-κB levels in control siRNA-treated cells divided by NF-κB levels of cells treated with siRNA against NIK. Higher ratios indicate more dependence on NIK to activate NF-κB. (C) 293T cells were infected with WT and mutant *Yersinia* for four hours and levels of the non-canonical NF-κB subunit p52 visualized via Western blot. As a control, 293T cells expressing BAFF receptor were treated with BAFF ligand to activate non-canonical NF-κB (Hildebrand et al., 2010). A representative Western blot is shown. (D) NF-κB luciferase reporter 293T cells were infected with various *Pseudomonas* strains for four hours and luminescence measured. (E) As in B, the experiment was repeated in the presence of control siRNA or siRNA against NIK. The average of at least three independent experiments ± SEM is shown. *p < 0.05, **p < 0.005, ***p < 0.0005, , as determined by one-way ANOVA with Tukey’s HSD post-hoc test, where each indicated group was compared to the appropriate negative and positive controls: (A and D) all strains compared, (B) Δyop6 and WT or Δyop6, ΔyopB, and WT, (E) ΔexoUT, ΔexoU, and ΔpopBD.
therefore blocking nuclear translocation of RelB/p52, prior to transfection with our NF-κB luciferase reporter and infection with *Yersinia*. As previously shown the Δyop6 strain induced higher levels of overall NF-κB activity than WT or the ΔyopB strain (Figure 3A). When NIK levels were knocked down, the Δyop6 strain triggered three-fold less NF-κB activity than in 293Ts treated with control siRNA (p = 0.003, Student’s t-test, Figure 3B), indicating the NF-κB response to the Δyop6 strain was primarily non-canonical.

WT *Y. pseudotuberculosis*, expressing YopHEMOJ, triggered less overall NF-κB activity, and activated NF-κB independently of NIK (Figures 3AB), suggesting that WT *Yersinia* instead triggers a canonical NF-κB response, unlike the Δyop6 strain. Expression of YopJ, an acetyltransferase known to dampen NF-κB activation (Bliska, 2006), was not sufficient to shift the response from canonical to non-canonical. While the a Δyop6 strain complemented with a plasmid encoding YopJ triggered less overall NF-κB activity than the parental Δyop6 strain (Δyop6+J; p = 0.01, Student’s t-test), the proportion of this activity that was dependent on NIK closely resembled that of the Δyop6 strain (p = 0.76, Student’s t-test; Figure 3AB). Collectively, these data show that the T3SS of the *Y. pseudotuberculosis* Δyop6 strain triggers non-canonical NF-κB activation, and suggest that Yop effectors other than YopJ shift the NF-κB response from the non-canonical to the canonical pathway in the WT strain.

**Yop effectors that target the actin cytoskeleton shift the host cell NF-κB response**
A strain translocating YopJ only, and no other effectors, caused a reduced overall NF-κB response. This response was through the non-canonical pathway, like that caused by the effectorless Δyop6 strain, while WT *Yersinia* triggered NF-κB through the canonical pathway. Since our WT *Yersinia* translocates YopJ and four other effectors, Yops EHMO, we aimed to determine which effector/s were necessary to shift the NF-κB response from non-canonical to canonical. We infected 293T cells with single-effector deletion mutants lacking Yops E, H, J, M, or O, and tested their NF-κB response (Figures 3AB). All five strains triggered canonical NF-κB at a similar level to WT *Y. pseudotuberculosis*, indicating that no single effector was responsible for shifting the NF-κB response from non-canonical to canonical.

Effector Yops E, H, and O have somewhat redundant functions, ultimately blocking actin polymerization and disrupting the actin cytoskeleton (Cornelis, 2006). We hypothesized these actin-targeting effectors were responsible for the different NF-κB responses between the WT and Δyop6 *Yersinia* strains. To test this, we assayed the ability of a *Y. pseudotuberculosis* strain lacking the actin cytoskeleton targeting effectors E, H, and O, but expressing Yops J and M, to activate NF-κB in 293T cells. The ΔyopEHO mutant induced overall NF-κB to the same level as WT *Y. pseudotuberculosis*, as the NF-κB-dampening effector YopJ is expressed in both strains (Figure 3A). However, the ΔyopEHO strain required NIK to activate NF-κB (p = 0.02, Student’s t-test; Figure 3B) like the Δyop6 strain. These results suggest that the actin-targeting effectors Yops E, H, and O are collectively required to shift the
NF-κB response from non-canonical to canonical, and that Yops J and M are dispensable for this effect.

The *Pseudomonas aeruginosa* T3SS triggers non-canonical NF-κB

To determine whether the non-canonical NF-κB response is unique to *Y. pseudotuberculosis*, or occurs in response to infection with other T3SS-expressing pathogens, we tested the NF-κB response to *Pseudomonas aeruginosa*. We compared PA103 strains lacking the T3SS effectors ExoU and ExoT (ΔexoUT), containing a nonfunctional T3SS (ΔpopBD), or containing only the actin-targeting effector ExoT (ΔexoU). WT *P. aeruginosa* could not be used in this assay as the phospholipase ExoU rapidly lysed host cells before the NF-κB response could be measured (data not shown).

*P. aeruginosa* ΔexoUT caused 4.5-fold more NF-κB activation than did the ΔpopBD strain, while ΔexoU did not induce a significant response (p = 0.29, ANOVA Tukey’s HSD post-hoc, Figure 3D). Like the Δyop6 *Y. pseudotuberculosis* strain, ΔexoUT triggered largely NIK-dependent NF-κB in contrast to the ΔpopBD or ΔexoU *P. aeruginosa* strains (Figure 3E). This data, along with Figures 3ABC, indicates that *P. aeruginosa* and *Y. pseudotuberculosis* strains lacking actin-targeting effectors induce the non-canonical NF-κB pathway, and that translocation of actin-modulating effectors into host cells switches this response to the canonical pathway.
**Actin-targeting effectors prevent *Y. pseudotuberculosis* invasion of host cells**

To further understand how the T3SS activates NF-κB, we investigated the ability of our *Y. pseudotuberculosis* strains to invade 293T cells. WT *Y. pseudotuberculosis* remained largely extracellular due to the activities of Yops E, H, and O (Figure 4). The Δyop6 strain invaded at the highest rate, with 62% of bacteria internalized two hours post-inoculation. The ΔyopEHO strain and the T3SS-deficient ΔyopB strain were unable to prevent internalization by host cells and displayed 51 and 55% invasion, respectively. These results suggest that internalization is necessary but not sufficient for non-canonical NF-κB activation, as the Δyop6, ΔyopEHO, and ΔyopB strains invaded, but only the Δyop6 and ΔyopEHO strains, containing a functional T3SS, triggered the non-canonical NF-κB response. This differential localization did not lead to differences in host cell survival, as infection with the WT, Δyop6, and ΔyopB strains did not cause significant LDH release compared to uninfected controls (Figure S2).
Figure 4. *Yersinia* Mutants that Cannot Target the Actin-Cytoskeleton are Internalized

(A) 293T cells were infected with WT and mutant *Yersinia* for two hours and infected cells visualized by confocal microscopy. Red bacteria are extracellular (left panels) whereas bacteria stained both red and green are intracellular (middle panels). 293T nuclei are stained with DAPI (blue). Representative images are shown. (B) The average percent of internalized bacteria of three independent experiments ± SEM is shown. *p < 0.005, as determined by one-way ANOVA with Tukey’s HSD post-hoc test, comparing all strains.
**Extracellular bacteria do not activate non-canonical NF-κB**

We hypothesized that location of T3SS activity, irrespective of effector presence, was the critical factor determining the type of NF-κB response. To test this, we pretreated 293T cells with cytochalasin D to prevent bacterial internalization, and quantified the type of NF-κB response following infection. Cytochalasin D pre-treatment of 293T cells infected with either *Y. pseudotuberculosis Δyop6* or *P. aeruginosa ΔexoUT* led to loss of dependence on NIK for NF-κB activation (Figure 5A). This suggests *Y. pseudotuberculosis* and *P. aeruginosa* must be internalized and also must possess a functional T3SS to activate non-canonical NF-κB. Since internal or external *Y. pseudotuberculosis* still translocate T3SS cargo into 293Ts (Figure 2B) (Duncan et al., 2014), these data also argue that the T3SS triggers the non-canonical NF-κB pathway through location-specific membrane perturbation rather than via the T3SS-mediated translocation of an unknown PAMP.

**Intracellular and extracellular T3SS-positive Yersinia activate distinct host signaling pathways**

To confirm that the *Y. pseudotuberculosis* WT and Δyop6 strains activate NF-κB through different mechanisms, we independently treated 293Ts with 17 out of the 24 compounds we obtained from our chemical genetics screen and measured NF-κB activation triggered by WT *Y. pseudotuberculosis* (Figure 5B). We found that eight compounds blocked both WT and Δyop6-mediated NF-κB activation while the remaining nine compounds did not significantly block WT-driven NF-κB activation.
Figure 5. Intracellular and Extracellular T3SS-positive Bacteria Trigger Distinct NF-κB Pathways. (A) NF-κB luciferase reporter 293T cells were treated with control siRNA or siRNA against NIK, infected with Yersinia or Pseudomonas in the presence or absence of the actin disrupting toxin cytochalasin D, and luminescence measured. The average of at least three independent experiments is shown ± SEM. (B) Inhibition of WT Yersinia-induced NF-κB activation by host-targeted molecules that blocked Δyop6-triggered NF-κB activation. NF-κB luciferase reporter 293T cells were infected with WT Y. pseudotuberculosis in the presence of one of a subset of the 24 compounds identified in Figure 1 for four hours and luminescence measured. The average of at least three independent experiments is shown ± SEM. (C) Model of NF-κB activation by extracellular and intracellular bacteria. Numbers (1, 2, 3) reference clusters of compounds listed in Table 2. Bacteria expressing a functional T3SS that remain extracellular trigger mainly a canonical NF-κB response, perhaps through β1-integrin signaling or Rho GTPase targeting effectors. In contrast, bacteria expressing a functional T3SS that become internalized induce a largely NIK-dependent NF-κB response. Lastly, bacteria not expressing a functional T3SS that become internalized trigger very little NF-κB activation. Cluster 1 compounds broadly inhibit T3SS function and therefore largely block both NF-κB-activating pathways. Cluster 2 compounds inhibit both NF-κB-activating pathways but do not interfere with general type III secretion. Cluster 3 compounds dampen NF-κB activation in response to internalized T3SS-positive bacteria but not in response to extracellular T3SS-positive bacteria, and the host pathways they target are predicted to be important for this
unique response. *p < 0.03, **p < 0.001, as determined by Student T-Test (A) or one-way ANOVA with Tukey’s HSD post-hoc test, where each indicated group was compared to the appropriate negative and positive controls (ΔyopB + DMSO and WT + DMSO).
These results further support that the WT and Δyop6 *Y. pseudotuberculosis* strains trigger different NF-κB pathways through distinct mechanisms.

We grouped the 17 compounds, all of which inhibited Δyop6-triggered NF-κB, into three clusters (Table 2). Cluster 1 contained five compounds that blocked either T3SS-mediated membrane perturbation or Yop translocation. Of these, three also blocked WT *Y. pseudotuberculosis*-induced NF-κB, suggesting that these compounds inhibit T3SS activity in both the WT and Δyop6 strains and therefore prevent both strains from inducing host responses. As WT *Y. pseudotuberculosis* expresses the actin-targeting effectors YopEHO, cytochalasin D had no further effect on NF-κB activity in response to WT *Y. pseudotuberculosis*. It is unclear why TPEN reduced Yop translocation but not WT *Y. pseudotuberculosis*-induced NF-κB activation. Cluster 2 contained compounds that did not inhibit T3SS-mediated membrane perturbation or Yop translocation, but inhibited both WT and Δyop6 *Y. pseudotuberculosis*-induced NF-κB activation. These data suggest that the host pathways involved in the response to the WT and Δyop6 strains may share common components, such as p38 and JNK mitogen activating protein (MAP) kinases. In contrast, cluster 3 contained compounds that did not inhibit T3SS-mediated membrane perturbation or Yop translocation, nor WT *Y. pseudotuberculosis*-induced (canonical) NF-κB activation. These compounds, which specifically block the ability of the *Yersinia* T3SS to induce non-canonical NF-κB activation, included several that
target lipid modification and vesicular trafficking, suggesting T3SS-mediated vacuolar disruption may be involved in triggering the non-canonical NF-κB pathway.

**DISCUSSION**

We used chemical genetics and RNA interference to elucidate the host NF-κB response to the bacterial T3SS. T3SS-positive extracellular *Y. pseudotuberculosis* and *P. aeruginosa* triggered NF-κB activation in a NIK-independent manner, indicative of the canonical NF-κB pathway in HEK293T cells. In contrast, internalized T3SS-positive *Y pseudotuberculosis* and *P. aeruginosa* induced NF-κB activation in a largely NIK-dependent manner, indicative of the non-canonical NF-κB pathway. The host response to extracellular bacteria was distinct from that induced by intracellular bacteria, as these pathways were sensitive to a different set of small molecules. These data suggest that mammalian cells are capable of distinguishing between the activities of a bacterial T3SS on the plasma membrane versus the vacuolar membrane.
Table 3. Impact of selected compounds on the *Yersinia* T3SS.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Inhibits Δyop6-driven NF-κB (%)</th>
<th>Inhibits WT-driven NF-κB (%)</th>
<th>Inhibits T3SS(^1)</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin</td>
<td>98%</td>
<td>87%</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>PP2</td>
<td>96%</td>
<td>31%</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>Cyclo[N-Arg...-Pro]</td>
<td>85%</td>
<td>46%</td>
<td>+</td>
<td>1</td>
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<td>TPEN</td>
<td>98%</td>
<td>No inhibition</td>
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<td>1</td>
</tr>
<tr>
<td>Cytochalasin D</td>
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<td>1</td>
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<td>KN-62</td>
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<td>77%</td>
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<td>2</td>
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<tr>
<td>FPL-64176</td>
<td>82%</td>
<td>31%</td>
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<td>Genistein</td>
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<tr>
<td>Cloprostenol</td>
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<td>-</td>
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<td>Swainsonine</td>
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<td>RH-80267</td>
<td>66%</td>
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<td>-</td>
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</tr>
<tr>
<td>Nocodazole</td>
<td>47%</td>
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<td>-</td>
<td>3</td>
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<tr>
<td>Wortmannin</td>
<td>86%</td>
<td>No inhibition</td>
<td>N.D.(^2)</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^1\) Compounds either significantly inhibited T3SS-mediated host membrane disruption or Yop translocation.

\(^2\) Wortmannin was incompatible with both T3SS assays due to autofluorescence and reduced HEK293T adherence to the plate. Wortmannin was grouped into cluster 3 due to a previous study showing wortmannin did not affect T3SS-mediated membrane disruption (Mejia et al., 2008).
We identified five compounds that inhibited either T3SS-mediated host membrane perturbation or effector protein translocation into host cells. Of these, several were previously described to reduce type III secretion by *Y. pseudotuberculosis*. Cytochalasin D, a fungal toxin that binds actin to prevent its polymerization, was shown to reduce T3SS-mediated host membrane disruption and YopE translocation into HeLa cells (Mejia et al., 2008) to similar levels observed in our study. That same study also demonstrated that PP2, an inhibitor of SRC-tyrosine kinases, blocked Yop translocation and bacterial internalization into HeLa cells. We also found that three additional compounds block translocation and/or pore formation: TPEN, cyclo[Arg-Gly-Asp-D-Phe-Val], and forskolin (Figure 2). Cyclo[Arg-Gly-Asp-D-Phe-Val] antagonizes integrins required for *Y. pseudotuberculosis* attachment to host cells through the bacterial adhesins invasin or YadA (Isberg and Leong, 1990; Yang and Isberg, 1993). As we have found YadA to be important for the ability of *Y. pseudotuberculosis* ∆yop6 to trigger NF-κB activation (data not shown), it is unsurprising that cyclo[Arg-Gly-Asp-D-Phe-Val] prevented effector translocation and T3SS-induced NF-κB activation. Previous studies have implicated β1-integrin engagement in T3SS-independent NF-κB activation, although we did not observe a significant NF-κB response to ∆yopB *Y. pseudotuberculosis* (Figure 3A) (Grassl et al., 2003; Schmid et al., 2004). Forskolin, a phospholipase C inhibitor, reduced T3SS-mediated membrane disruption but not translocation. Accordingly, the ability of the *Yersinia* T3SS to carry out host membrane perturbation and to translocate T3SS cargo into target host cells have been shown to be genetically separable activities (Adams et
al., 2015; Costa et al., 2010; Kwuan et al., 2013; Olsson et al., 2004; Zwack et al., 2015). Uliczka et al. found phospholipase C to be required for *Yersinia* invasion of HEP-2 cells (Uliczka et al., 2009), so it is also possible that forskolin also inhibits bacterial uptake in HEK293Ts and this prevents activation of non-canonical NF-κB. TPEN, a zinc chelator, modestly reduced translocation into host cells, but did not significantly inhibit T3SS-mediated membrane perturbation. Recently, Li et al. implicated zinc in altering levels of LcrF, the *Yersinia* T3SS master regulator (Li et al., 2014). However, we did not observe an effect of TPEN on *Yersinia* type III secretion *in vitro* (data not shown). Therefore, the effects of this zinc chelator on the *Yersinia* T3SS remain unclear.

Of the 17 compounds selected for further study from our chemical genetics screen, 11 blocked the host NF-κB response without affecting type III secretion. Five of these (cluster 2 in Table 2) blocked both Δyop6- and WT-induced NF-κB and are primarily composed of kinase inhibitors. These results suggest that kinases, including p38 and JNK MAP kinases, which are inhibited by compounds in cluster 2, are required for the NF-κB response to both WT and effectorless bacteria. Viboud et al. have proposed that the *Yersinia* YopBD translocon triggers activation of MAP kinases and canonical NF-κB (Solomon et al., 2015; Viboud et al., 2006). Solomon et al. showed that HeLa cells infected with *Y. pseudotuberculosis* lacking YopEHJ contained more of the canonical NF-κB inhibitor IκBα than did mutants lacking either YopD or YopB (Solomon et al., 2015). As the ΔyopEHJ strain expressed the actin-targeting effector
YopO, it is unclear whether it was internalized. However, our data would predict the ΔyopEHJ strain remains extracellular given the apparent canonical NF-κB response. Another compound that blocked both Δyop6 and WT-induced NF-κB was KN-62, a calmodulin-dependent kinase type II (CaM KII) inhibitor. Sheahan and Isberg recently identified calmodulins as hits in their shRNA screen designed to uncover genes important for Y. pseudotuberculosis T3SS-mediated Yop translocation or YopE effector function (Sheahan and Isberg, 2015). As in our work, they found blocking calmodulins did not affect T3SS-mediated membrane perturbation in 293T cells, but it is unclear how calmodulins might be important for translocation, YopE function, and/or NF-κB activation.

The remaining seven bioactive molecules (cluster 3 in Table 2) specifically inhibited Δyop6-, but not WT-, induced NF-κB and primarily affected lipid modification and vesicular trafficking. For example, nocodazole inhibits microtubule polymerization and has been shown to reduce Y. pseudotuberculosis invasion of HeLa cells (McGee et al., 2003). Phosphatidylinositol 3-kinases, blocked by wortmannin, have a variety of cellular roles relating to proliferation, motility, and growth. Relevant to our work is the enzyme’s involvement in cellular trafficking of hydrolases to lysosomes and phagosomal biogenesis (Davidson, 1995; Fratti et al., 2001). Compounds of cluster 3, which do not affect type III secretion or canonical NF-κB (induced by external, T3SS-positive bacteria), primarily target vesicular trafficking and lipid modification.
This supports our conclusion that internalized bacteria trigger NF-κB through vacuolar membrane disruption.

Borderline hits can provide valuable information, especially in the context of other bioactive molecules used in the screen. For instance, three protein kinase C (PKC) inhibitors reduced T3SS-driven NF-κB to a greater extent than did TNF-α-driven NF-κB (Supplementary Dataset 1), although the data was only statistically significant for Gö6976 (p = 0.016, Student t-test). The 15 PKC isozymes are grouped into three subfamilies: conventional, novel, or atypical, based on their reliance on diacylglycerol, calcium, and/or phospholipids for activation. The “borderline hit” molecules GF 109203X, Gö6976, and HBDDE are all specific for the conventional class of PKCs, inhibiting the α, β, and γ isozymes. Furthermore, RHC-80267, a hit in cluster 3, inhibits diacylglycerol lipase and phospholipase C (PLC), enzymes that act upstream of conventional PKC activation. Interestingly, Ashida and coworkers recently described the involvement of PKC in sensing vacuolar membrane disruption due to translocated T3SS effectors from internalized Shigella flexneri (Ashida et al., 2013). This response was dependent on the novel class of PKCs and led to NF-κB activation in HeLa cells. While we did not find a role for the novel class of PKCs in Yersinia T3SS-induced NF-κB activation, perhaps a conventional isozyme of PKC may be involved in sensing T3SS-mediated vacuolar membrane damage.
We found that internalized *Y. pseudotuberculosis* and *P. aeruginosa* trigger a distinct NIK-dependent NF-κB pathway. This non-canonical NF-κB activation was dependent on a functional T3SS, as pore formation mutants (ΔyopB and ΔpopBD) were internalized but did not induce NF-κB. If cytochalasin D was used to force bacteria lacking actin-targeting effectors to remain extracellular, the bacteria no longer activated NF-κB in a NIK-dependent manner. Yet cytochalasin D still allowed 60% of normal Yop translocation, suggesting it does not completely block translocation of T3SS cargo into the host cytosol. Collectively, these data suggest that host cells sense vacuolar T3SS-mediated membrane perturbation, rather than translocation of a bacterially-derived PAMP, leading to non-canonical NF-κB activation. The ability of mammalian cells to specifically detect the presence of a functional T3SS on an internalized membrane could represent a mechanism to distinguish between intra- and extracellular T3SSs.

We have shown that two T3SS-expressing pathogens, *Yersinia* and *Pseudomonas*, trigger non-canonical NF-κB activation and we speculate other bacteria, such as *Salmonella* spp. and *Chlamydia* spp., may cause this response but counter it with T3SS effector proteins. Hyperinduction of non-canonical NF-κB can lead to autoimmune disorders and rheumatoid arthritis (Brown et al., 2008). Curiously, several of the main causes of reactive arthritis, a form of acute arthritis following gastrointestinal or urogenital infection, are *Yersinia* spp., *Chlamydia* spp. *Salmonella* spp., *Shigella* spp. and *Campylobacter jejuni* (Selmi and Gershwin, 2014). These
pathogens all use T3SSs (*Campylobacter* can use its flagella as a T3SS (Konkel et al., 2004)), and it is tempting to speculate that long-lasting T3SS-mediated non-canonical NF-κB activation could contribute to this autoimmune condition. Furthermore, persistent elevation of NIK levels is linked to multiple myeloma and colon tumorigenesis, suggesting the possibility that chronic infection by T3SS-expressing pathogens may put patients at a higher risk for certain cancers (Allen et al., 2012; Demchenko et al., 2010; Keats et al., 2007).

Previous studies have elucidated independent mechanisms by which host cells recognize the T3SS. There is evidence that translocation of T3SS needle and inner rod subunits through the T3SS into host cells leads to inflammasome activation via NAIP PRRs (Bergsbaken and Cookson, 2007; Kofoed and Vance, 2011; Schotte et al., 2004; Shin and Cornelis, 2007). In addition, Keestra *et al.* recently demonstrated that the downstream activities of a *Salmonella* Rho GTPase-activating effector could be sensed by Nod1, leading to induction of NF-κB and MAPK pathways (Keestra et al., 2013). Our previous data showed that the NF-κB response to the effectorless *Y. pseudotuberculosis* Δyop6 strain was independent of Nod1 and Nod2 (Auerbuch et al., 2009). Our findings suggest that a distinct pathway senses the presence of a functional T3SS deployed by an internalized pathogen, leading to NIK-dependent NF-κB activation. This previously unappreciated response could have implications for infection by vacuolar pathogens or by primarily extracellular pathogens such as *Yersinia* and *Pseudomonas* that are not 100% efficient at inhibiting internalization.
Supplemental Figure 1.

Gating strategy for flow cytometry-based translocation assay (Figure 2B). Cells in the green gate contain uncleaved CCF2-AM. Cells in the blue gate contain cleaved CCF2-AM, indicating the presence of the T3SS-translocated YopH-β-lactamase fusion protein. Cells in the aqua gate contain cleaved and uncleaved CCF2-AM, and were not used for quantification.
Supplemental Figure 2.

293T cells were infected with WT, Δyop6, or ΔyopB Yersinia for four (A) or 24 hours (B) and cytotoxicity measured by lactate dehydrogenase (LDH) release. The average of three independent experiments ± SEM is shown.
EXPERIMENTAL PROCEDURES

Bacterial strains and cell lines

The *Y. pseudotuberculosis* IP2666 and *P. aeruginosa* PA103 strains used in this study are listed in Table S1. The *Y. pseudotuberculosis* ΔyopH and ΔyopO deletions were constructed as previously described (Miller et al., 2014), using primer pairs designed using Primer 3 software (http://fokker.wi.mit.edu/primer3/input.htm): F5’yopO (CGGTGAATGGGGATACAAAG), R5’yopO (TAGGGGGCACTTGTCACATCCCCATGATTTCACGCTTTT), F3’yopO (AAAAGCGTGAAAATCATGGGGATGTGACAAGTGCCCCCTA), R3’yopO (TGGAGAAATGGCAATCAGGT), F5’yopH (CGCCAGACATTCACGACTAA), R5’yopH (TTGATTGGCAAGTGGTTTTTAATAATAGGTGAGCCGTGT), F3’yopH (GGCTCACCTATTATTAAAAACCACCTTGCCAATCAAGAA), R3’yopH (ACCACTGCTGGTTCAGTCGAT).

HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO₂.

General Growth/ Infection Conditions

*P. aeruginosa* were grown overnight in Luria broth (LB) at 37°C with shaking. On the day of infection, cultures were backdiluted to an OD₆₀₀ of 0.2 into LB and
incubated at 37°C with shaking for 3 hours. Cultures were normalized for OD$_{600}$ and added to eukaryotic cells for infection.

*Y. pseudotuberculosis* cultures were grown overnight in 2xYT media at 26°C with shaking. On the day of infection, the cultures were backdiluted to an OD$_{600}$ of 0.2 into low calcium media and incubated at 26°C with shaking for 1.5 hours. The cultures were then shifted to 37°C with shaking for 1.5 hrs. Cultures were normalized for OD$_{600}$ and added to eukaryotic cells for infection.

**Screen For Bioactive Inhibitors of T3SS-induced NF-κB**

HEK293T cells were transfected, plated, infected, and their luminescence measured as previously described (Duncan et al., 2014). Immediately prior to infection, compounds from the ICCB Known Bioactives library (ENZO) or DMSO vehicle control were added to the 384-well plate containing 293T cells by a pinning robot (Janus MDT, PerkinElmer).

The secondary screen for inhibitors of TNF-α-induced NF-κB was performed similarly to that the original screen for inhibitors of T3SS-induced NF-κB activation except instead of adding bacteria, 1.5ng/µl final concentration of TNF-α (Invivogen) was added to each well of the 384-well plate containing 293T cells. On average, TNF-α induced 42 fold more luminescence than in the untreated wells (data not shown).
Translocation Assay

5.2x10^5 293T cells were plated in each well of a 24-well plate in 1 ml DMEM plus 10% FBS on the day of the infection. One hour prior to infection, 2.85 µl compound or DMSO was added to each well. Cells were infected at MOI 4 for 1 hour at 37°C/5% CO₂. CCF2-AM (Invitrogen) was added and the cells were incubated in the dark for 30 minutes at room temperature. The cells were resuspended in DMEM, pelleted for 2 minutes at 4000xg, resuspended in 4% paraformaldehyde for 10 min, pelleted for 2 min at 4000xg, and finally resuspended in PBS. Fixed cells were analyzed for blue (cleaved CCF2) and green (uncleaved CCF2) fluorescence on an LSRII flow cytometer (Becton Dickson). At least 10,000 cells were acquired per sample and data was analyzed using FlowJo v8.8.7 software. Flow cytometry gates (Figure S1) for green and blue fluorescence were set using uninfected 293T cells (negative control) and 293T cells infected at MOI 200 (positive control).

Western Blot

To assess processing of p100 to p52, 293T cells were plated at a density of 2x10^6 cells per well of a 6-well plate and incubated overnight. The next day, the cells were infected with Y. pseudotuberculosis at MOI 15 for 4hrs. The cells were lysed with 1% digitonin and lysates run on a 7.5% SDS-PAGE gel. Following semi-dry transfer to immobilon-P, samples were analyzed by Western blotting for p100/p52 (Millipore).
Densitometric quantification of the bands was performed using Image Lab software (Bio-Rad).

**RNA Interference**

3x10^4 293Ts cells were plated in each well of a 24-well plate in 400 µl DMEM plus 10% FBS and incubated overnight. On day two, the cells were transfected with siRNA targeting NIK or scrambled control siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The following day the cells were transfected with an NF-κB luciferase reporter plasmid (Stratagene) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. On day four, the 293Ts were infected with *Y. pseudotuberculosis* at MOI 7. Following a four hour infection at 37°C/5% CO2, the medium was aspirated before adding a 1:1 Neolite–phosphate-buffered saline (PBS) solution. Plates were covered in foil, incubated for 5 min, and bioluminescence measured using an EnVision plate reader (PerkinElmer).

**Ethidium Bromide Entry Assay**

96-well clear-bottom black plates were pre-treated with Poly-L Lysine (Sigma) to improve cell attachment. 2x10^4 293T cells were plated in each well of a 96-well plate in 100 µl DMEM plus 10% FBS and incubated overnight. The 293Ts were preincubated with chemicals or DMSO for 1 h prior to infection. The cells were infected in triplicate at MOI 25 and centrifuged for 5 min at 113xg at 4°C to initiate
contact. The cells were then incubated at 37°C/5% CO2 for 1 h. At the end of the incubation period, the medium was aspirated and replaced with 30 µl of PBS containing 25 µg/ml ethidium bromide (EtBr) and 5 µM DRAQ5 dye (Cell Signalling). The cell monolayer was visualized using an ImageXpressMICRO automated microscope and MetaXpress analysis software (Molecular Devices). The percentage of EtBr-positive cells was calculated by dividing the number of EtBr-stained cells by the number of DRAQ5-stained cells. Data from three separate wells were averaged for each of three independent experiments.

**LDH Release**

4.2x10^4 HEK293T cells were plated in each well of a 96-well plate in 100 µl DMEM plus 10% FBS and incubated overnight. The following day the cells were infected in triplicate at MOI 7. The cells were then incubated at 37°C/5% CO2 for 4 or 24 hours. For the 24-hour time point, chloramphenicol was added at four hours post-inoculation. Following infection, the supernatant was transferred to microcentrifuge tubes and centrifuged to pellet cellular debris. For full cell lysis, 3 wells were flash frozen. 50 µl of the supernatant was then transferred to a new 96-well well plate, and incubated for 30 minutes with 50 µl of substrate mix (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega). Following the incubation, 50 µl of stop solution was added to each well, and the absorbance measured at 490nm. The fully lysed wells were averaged and percent LDH release was calculated for each infection condition.
Invasion Assay

12mm round coverslips were autoclaved and treated with sterile Poly-L lysine (Sigma) diluted 1:10 in water for 5 min. Coverslips were washed with sterile water and allowed to dry for at least 2 hours before use. 2.6x10^5 293T cells were plated in each well of a 96-well plate in 400 µl DMEM plus 10% FBS and incubated overnight. The following day, the 293T cells were infected at MOI 7 for two hours. After 30 min, cell media was aspirated and replaced with fresh media to remove non-attached cells. To end the infection, cells were fixed with 4% formaldehyde for 10 min and incubated with an anti-Yersinia antibody (generously provided by R. Isberg) followed by an anti-rabbit Alexa-fluor 594 secondary antibody (Life Technologies). The 293T cells were then permeabilized with ice-cold methanol for 10 sec, incubated with the anti-Yersinia antibody followed by an anti-rabbit FITC secondary antibody (Santa Cruz Biotechnology) and Hoechst diluted 1:10,000 in PBS, mounted using ProLong Gold mounting media (Life Technologies), and imaged using a Leica SP5 Confocal Microscope. Approximately 300 bacteria were counted per strain for each of three biological replicates.

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